Effect of exogenous fibrolytic enzymes on fibre and protein digestion in ruminant animals

by

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Declaration

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Abstract

Title: The effects of exogenous fibrolytic enzymes on fibre and protein digestion in ruminant animals

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Forages are the main feed components in ruminant production systems for the reason that they are often the major source of energy available to the animal. However, only 10 to 35% of energy intake is available as net energy because the digestion of plant cell walls is not complete. This can significantly affect livestock performance and profits in production systems that use forages as a major source of nutrients of the diet. As a result of low and variable nutritive values of forage feedstuffs, attempts to improve ruminal fibre degradability have been an ongoing research topic. The use of exogenous fibrolytic enzymes (EFE) has been proposed as means to improve forage digestibility. Positive results with regard to rumen forage digestibility and other animal production traits have consequently been obtained due to increased rumen microbial activity following EFE addition in ruminant diets.

Two EFE (Abo 374 and EFE 2) and one commercial yeast preparation were firstly identified and selected for their potential to improve the cumulative gas production (GP) at 24 hours of a range of feed substrates using the *in vitro* GP system as a screening step to identify the superior EFE products. The different feed substrates were lucerne hay, wheat straw, wheat straw treated with urea and a commercial concentrate diet. An *in vitro* experiment was undertaken on these four different substrates in order to evaluate the two EFE and the yeast preparation. This was to identify the most promising EFE capable of producing a significant effect on feed digestibility using organic matter digestibility (*in vitro* true digestibility) and fermentation characteristics (*in vitro* GP system). Results from the *in vitro* evaluation showed that EFE significantly enhanced *in vitro* DM degradability and GP profiles ($P < 0.05$). Abo 374 enzyme showed potential to increase *in vitro* microbial protein synthesis (MPS) of GP residues of the concentrate diet. In addition, no correlation was found between the *in vitro* MPS and the 48 hours cumulative GP of all the tested substrates ($P < 0.05$; $R^2 < 0.30$). Treatments were found to increase *in vitro* MPS, feed degradability and the cumulative GP of different quality forages and the concentrate diet, with Abo 374 being the best treatment ($P < 0.05$). However *in vitro* responses of EFE were variable depending on the energy concentration and chemical composition of different substrates. Variation in MPS was mostly due to the low recovery of purine derivates with the purine laboratory analysis.

On the basis of these results, Abo 374 was selected and consequently further tested in another *in vitro* and *in situ* trial using a mixed substrate of lucerne hay and wheat straw. Abo 374 significantly improved the cumulative GP, *in vitro* DM and NDF disappearance of the mixed substrate ($P < 0.05$). In addition, no correlation was found between the *in vitro* MPS and the cumulative GP at 48 hours ($P = 0.68$; $R^2 < 0.25$). The *in situ* disappearance of feed nutrients (DM, NDF and CP) with Abo 374 was similar to the control. The lack of significance of disappearance was probably due to the small number of sheep used in the study and the relatively high coefficient of variation associated with measuring ruminal digestion. Abo

374 significantly increased the *in situ* MPS (P = 0.0088) of the mixed substrate of lucerne hay and wheat straw. Evidence of the increased MPS and both *in vitro* and *in situ* disappearance of DM and NDF resulted from the Abo 374 activity during either the pre-treatment or the digestion process. The addition of Abo 374 to the mixed substrate of lucerne hay and wheat straw appeared to have been beneficial for microbial colonization of feed particles as a result of the increased rumen activity. It could be speculated that the primary microbial colonization was thus initiated, leading to the release of digestion products that attract in return additional bacteria to the site of digestion. This EFE may be efficient to produce some beneficial depolymerisations of the surface structure of the plant material and the hydrolytic capacity of the rumen to improve microbial attachment and the feed digestibility thereafter. Therefore, the mechanism of action by which Abo 374 improved the feed digestion can be attributed to the increased microbial attachment, stimulation of the rumen microbial population and synergistic effects with hydrolases of ruminal micro-organisms. With regard to these findings, the addition of EFE in ruminant systems can improve the ruminal digestion of DM, NDF and CP to subsequently enhance the supply of the metabolizable protein to the small intestine.

Key words: crude protein (CP), exogenous fibrolytic enzymes (EFE), dry matter (DM), gas production (GP), neutral detergent fibre (NDF), microbial protein synthesis (MPS).

Uittreksel

Titel: Die invloed van eksogene fibrolitiese ensieme op vesel- en proteïen vertering in herkouende diere.

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Ruvoere is die hoof-voerkomponent in herkouer produksiesisteme aangesien dit dikwels die vernaamstebron van energie aan herkouer is. Slegs 10 tot 35% van die energie-inname is beskikbaar as netto-enrgie, omdat die vertering van selwande onvolledig is. Dit kan die prestasie en profyt in produksiesisteme drasties beïnvloed waar ruvoere as 'n hoofbron van nutriënte in die dieet gebruik word. Aangesien die nutriëntwaarde van ruvoere laag is en baie varieer, is navorsing vir verbeterde ruminale veselvertering steeds 'n voorgesette onderwerp. Dit is voorgestel dat eksogeniese fibrolitese ensieme (EFE) gebruik kan word vir verbeterde ruvoervertering. Positiewe resultate in ruminale ruvoerverterig en ander diereproduksie-eienskappe, is verkry as gevolg van toenemende rumen mikrobiese aktiwiteit na EFE aanvulling in herkouerdiëte.

Twee EFE's (Abo 374 en EFE 2) en `n gisproduk is geïdentifiseer en geselekteer vir hul potensiaal om die kumulatiewe gasproduksie (GP) na 24 uur met 'n reeks voersubstrate te verbeter met die gebruik van die *in vitro* GP sisteem as seleksiemetode om die superieure EFE produkte te identifiseer. Die verskillende ruvoersubstrate was lusernhooi, koringstrooi, ureumbehandelde koringstrooi en 'n kommersiële konsentraatdieet. 'n *In vitro* eksperiment was onderneem om die vier verskillende substrate te gebruik om die twee EFE's en gisproduk te evalueer. Hierdeur sou die belowendste EFE's identifiseer kon word wat 'n betekenisvolle effek op ruvoervertering het. Die vertering van ruvoer sal bepaal word deur organiese materiaal vertering (*in vitro* ware vertering), asook fermentasie-eienskappe (*in vitro* GP sisteem). Resultate van die *in vitro* evaluering het getoon dat EFE's *in vitro* DM degradering en GP profiele verbeter. Dit blyk dat die Abo 374 ensiem 'n potensiële toemame in *in vitro* mikrobiese proteïensintese (MPS), soos bepaal deur die GP oorblyfsels van konsentraat diëte, tot gevolg gehad het. Daar was geen korrelasie tussen die *in vitro* GP en MPS van al die proefsubstrate nie. Dit blyk dat die behandelings 'n toename in *in vitro* GP, MPS en ruvoerdegradeerbaarheid van lae kwaliteit ruvoer- en konsentraatdiëte gehad het, waar Abo 374 die beste behandeling was. Die *in vitro* reaksies van die EFE's was egter wisselend, afhangende van die energiekonsentrasie en die chemiese samestelling van die verskillende substrate. Variasie van MPS was meestal as gevolg van die lae herwinning van purienderivate tydens die purienanalise.

Op grond van dié resultate, is Abo 374 geselekteer om verdere toetse in ander *in vitro* en *in situ* proewe te doen. Die substraat wat gebruik is, was 'n 1:1 mengsel van lusernhooi en koringstrooi. Abo 374 het die kumulatiewe RP, *in vitro* DM en NBV verdwyning van die gemengde substraat verbeter. Boonop is geen korrelasie tussen die MPS en *in vitro* GP gevind nie. *In situ* verdwyning van DM, NBV en RP was hoër vir Abo 374, maar nie betekenisvol nie. Die gebrek aan betekenisvolle verdwynings mag die gevolg wees van die klein hoeveelheid skape wat in die proef gebruik is, asook die relatiewe hoë koëffisient van variasie wat gepaard gaan met die bepaling van ruminale vertering. Abo 374 het die *in situ*

MPS betekenisvol verhoog. Verhoogde MPS en *in vitro* en *in situ* verdwyning van DM en NBV is waargeneemwaarskynlik as gevolg van die aktiwiteit van Abo 374 gedurende die voorafbehandeling óf die verterings proses. Die byvoeging van Abo 374 tot die gemengde substraat van lusernhooi en koringstrooi blyk om voordelig te wees vir mikrobiese kolonisering van voerpartikels as gevolg van ’n toename in rumenaktiwiteit. Die primêre mikrobiese kolonisering het waaarskynlik gelei tot die vrystelling van verteringsprodukte wat addisionele bakterieë na die plek van vertering lok. Die EFE mag geskik wees vir voordelige depolimerisasie op die oppervlakstruktuur van die plantmateriaal, asook verbeterde hidrolitiese kapasiteit van die rumen om sodoende mikrobiese aanhegting, asook ruvoervertering te verbeter. Dus, Abo 374 se meganisme van aksie wat verbeterde ruvoervertering tot gevolg het, kan toegeskryf word aan `n verhoogde mikrobiese aanhegting, stimulering van die rumen mikrobiese populasie en die sinergistiese effek met hidrolases van rumen mikroörganismes. Ten opsigte van die bevindings, kan die byvoeging van EFE in herkouersisteme ruminale vertering van DM, NBV en RP verbeter, wat dan daaropvolgend die dunderm met meer metaboliseerbare proteïn sal voorsien.

Sleutelwoorde: eksogene fibrolitiese ensieme (EFE), droëmaterial (DM), ruproteïen (RP), neutraal bestande vesel (NBV), mikrobiese proteïensintese (MPS), gasproduksie (GP).

List of Abbreviations

AA: Amino acids
ADF: Acid-detergent fibre
ANOVA: Analysis of variance
ANF: Antinutritional factors
CH_4: Methane
CO_2: Carbon dioxide
CFU: coliform units
CP: Crude protein
DM: Dry matter
DMI: Dry matter intake
DMD: Digestible dry matter
EFE: Exogenous fibrolytic enzymes
GP: Gas production
H_2: Hydrogen
H_2O: Water
HCl: Chloride hydrogen
MPS: Microbial protein synthesis
NDF: Neutral-detergent fibre
N: Nitrogen
NPN: Non protein nitrogen
NSP: Non starch polysaccharides
NSC: Non structural carbohydrates
P: Phosphor
O_2: Oxygen
OM: Organic matter
OMD: Digestible organic matter
P: Probability
RDP: Rumen degradable protein
RNA: Ribo nucleic acid
RUP: Rumen undegradable protein
SU ACUC: Stellenbosch University animal care and use committee
TMR: Total mixed ration
VFA: Volatile fatty acids

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Figure 6.6 Microbial protein synthesis measured as purine derivates (RNA equivalent in µg/g DM) on residues of *in situ* nylon bag digestion of the mixed substrate of lucerne hay and wheat straw.

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Dedication

I have seen something else under the sun:
The race is not to the swift
or the battle to the strong,
nor does food come to the wise
or wealth to the brilliant
or favour to the learned;
but time and chance happen to them all.
Ecclesiastes 9: 11

To whom, by grace, makes the impossible possible,
To whom my success and progress are their major concern,
I dedicate this work.

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CHAPTER 1

General introduction

Ruminant animals may be considered as the foundation of animal agriculture because they have served mankind all the way through many millennia (Weimer *et al.*, 2009). The ruminant production systems are dependant worldwide on forage as the main nutritional components (Wilkins, 2000). The digestion of forage occurs through the microbial fermentation as a result of the presence of the reticulorumen and its adaptation to digest lignocellulosic components. The microbial mode of digestion allows ruminants to better unlock the unavailable energy in the plant cell wall components than other herbivores (Van Soest, 1994; Krause *et al*., 2003). This gives ruminant animals the ability to convert low nutritive and resistant lignocellulosic biomass to milk, meat, wool and hides (Weimer *et al*., 2009). However, most forage plants are high in cell walls and low in nitrogen (N) and energy content (Romney & Gill, 2000). Despite the importance of fibrous components in forages for salivation, rumen buffering and efficient production of ruminal end products (Mertens, 1997), only 10 to 35% of energy intake is available as net energy (Varga & Kolver, 1997). This is because the ruminal digestion of plant cell walls is not complete (Krause *et al*., 2003). Furthermore, tropical pastures are always of low yield and variable quality due to climate constraints. With the effect of temperature and shortage of precipitation, most available natural C4 grass pastures and crop residues are of poor nutritive value as they consist of highly lignified stems during the dry season (Meissner, 1997). Consequently, performance of ruminants fed such feedstuffs as major components of nourishment is often suboptimal because of their high lignin concentrations. Cross linkages formed between ferulic acid and lignin, which increase with age, limit the microbial access to the digestible xylans in the cell wall networks of plants (Krueger *et al*., 2008)

As a consequence of a low nutritive value of forage at maturity, many strategies have been developed to improve the nutritional quality of forages used in ruminant systems. These have consisted of the plant breeding and management for improved digestibility (Casler & Vogel, 1999) and the increase of feed utilization by physical, chemical and/or biotechnological actions (McDonald *et al*, 2002). Despite improvements in cell wall digestibility achieved through these strategies, forage digestibility continues to limit the intake of digestible energy in ruminants because not even 50% of this fraction is readily digested and utilized (Hatfield *et al*., 1999). Investigations on the attempts to improve forage utilization remained an important area of research in animal production for over a century. Large quantities of biologically active enzymes as animal feed additives are now produced at low cost since recent improvements in fermentation technology and biotechnology. It is acknowledged that enzyme preparations with specific activities can be used to drive specific metabolic and digestive processes in the gastrointestinal tract and may increase natural digestive processes to improve the availability of nutrients and feed intake thereafter (Dawson & Tricarico, 1999; McAllister *et al*., 2001; Colombatto *et al*., 2003).

The use of biotechnology such as exogenous fibrolytic enzymes (EFE) to enhance quality and digestibility of fibrous forage is on the verge of delivering practical benefits to ruminant production systems. In this regard,

cellulases and xylanases are respectively amongst the two major enzyme groups that are specified to break ß1-4 linkages joining sugar molecules of cellulose and xylans found in plant cell wall components (Dawson & Tricarico, 1999; Beauchemin *et al*., 2003). Several studies with EFE have made mention of the increase of microbial activities in the rumen, which resulted in an enhancement of animal performance traits. Despite the increase in feed digestibility and subsequent production traits, the relationship between the improvement in forage utilization and enzymatic activities is yet to be explained in ruminant systems (Eun *et al*., 2007). In addition, results with EFE addition in ruminant systems are variable and somewhat inconsistent (Beauchemin *et al*., 2003; Colombatto *et al*., 2003), making their biological response difficult to predict. Some studies have shown substantial improvement of feed digestibility and animal performance traits (Lewis *et al*., 1999; Rode *et al*., 1999; Yang *et al*., 1999 ; Nowak *et al*. 2003; Cruywagen & Goosen 2004; Bala *et al*., 2009), while others reported either negative effects or none at all (Vicini *et al*., 2003; Bowman *et al*., 2003; Baloyi, 2008).

Most EFE investigations in ruminant systems are aimed at enhancing the degradation of plant cell wall components (Eun & Beauchemin, 2007) due to their antinutritional effect in the diet. Amongst these studies, only few tended to evaluate the effect of EFE on protein digestion and microbial protein synthesis (MPS) (Yang *et al*., 1999; Giraldo *et al*., 2007a, b; Peters *et al*., 2010). The possible effect of EFE in animal nutrition is that improved fibre degradation can increase the energy concentration and the release of fibre-trapped nutrients (protein amongst others) of the diet (Bedford, 2000; Sheppy, 2001). This can improve the degradation of crude protein (CP) and also enhance MPS (Yang *et al*., 1999), total microbial population (Nsereko *et al*., 2002) and nitrogen (N)-fraction production in the rumen (Giraldo *et al*., 2007a, b). If the potential intake and/or the density of available nutrients of forages can be increased with EFE as feed additives, then poor quality forages can be economically and successfully converted into meat and milk for human consumption. This may contribute to low cost productions in ruminant systems using poor quality forages as major components.

Against this background, the objective of the current study was to revaluate the effects of EFE (Abo 374, EFE 2) on crude protein and fibre digestion in the ruminant system. Specific objectives were firstly to evaluate EFE for their impact on microbial protein synthesis (MPS) and the ruminal digestion of DM, NDF and CP using the GP profiles and the *in vitro* filter bag technique. It was also to determine the relationship between MPS and the cumulative GP at 48 hours of incubation. Secondly, the superior EFE identified from the previous investigation was further tested for its effects on the digestion of CP and the disappearance of DM and NDF to subsequently increase MPS in a parallel *in vitro* and *in situ* evaluation using cannulated Döhne-Merino sheep.

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CHAPTER 2

Literature review

A. Forages and ruminant nutrition

Cattle, sheep and goats play an important role in agriculture. They are able to convert low quality feeds into food of high biological value for human beings. This is because they are adapted to utilize plant cell walls as major component of nourishment (McDonald *et al*., 2002). The economic implications of forage cell walls in ruminant nutrition are undisputable. In the form of long particles, it is essential to stimulate rumination. This enhances the breaking down and the fermentation of fibrous components and stimulates the rumen contraction. Ruminating also maintains the rumen pH through buffer content in the saliva flow and cation exchange on the surface of fibre particle (acidosis prevention). As a result of this, great conditions are established in the rumen whereby indispensable end-products of fermentation are highly produced and absorbed for normal animal metabolism (Van Soest, 1991).

Plant cell walls found in forage feedstuffs are needed in ruminant daily intake, especially in dairy cows. These components determine the milk fat percentage, which is the production indicator for the well being animal and performance (Mertens, 1997). Furthermore fibrous components have nutritional effects of binding and removing potential harmful compounds such as constipation agents and carcinogen agents through faeces (McDougall *et al*., 1996). When insufficient coarse fibrous diet with high grain or less forage is fed, the rumen pH falls and the efficiency of digestion is compromised. This is because of the accumulation of organic acids (volatile fatty acids and lactic acid) and reduction of buffering capacity of the rumen (Plaizier *et al*., 2009). For that reason, an accurate daily fibre content will therefore prevent any economical loss from digestive and metabolic disorders leading sometimes to death. These disorders include: erosion of rumen epithelium, abscesses and inflammations of livers, milk fat depression, metabolic changes leading to fattening, diarrhea, acidosis causing ruminal parakeratosis and chronic laminitis, altered ruminal fermentation, reduced energy intake, etc. (Mertens, 1997; Plaizier *et al*., 2009).

1. Chemistry and structure of plant cell walls

Plant cell walls are complex biological structures that consist of polysaccharides (Table 2.1). These are associated with protein matrix (extensins) and phenolic compounds in the cell networks, together with lignin (Fisher *et al*., 1995; Knudsen, 2001; Graminha *et al*., 2008). According to the chemical definition, fibrous components represent the sum of non starch polysaccharides (NSP) and lignin (Theander *et al*, 1994) while physiologically they are known as the components that resistant to degradation by mammalian enzymes (McCleary, 2003).

Table 2.1 Constituents of dietary fibre (Source: De Vries, 2003).

NSP and resistant oligosaccharides	Analogous carbohydrates	Lignin substances associated with the NSP and lignin complex in plants
Cellulose	Indigestible dextrins	Waxes
Hemicellulose	Resistant maltodextrins *(from maize and other sources)*	Phytate
Arabinoxylans	Resistant potato dextrins	Cutin
Arabinogalactans	Synthesized carbohydrate compounds	Saponins
Polyfructoses	Polydextrose	Suberin
Inulin	Methyl cellulose	Tannins
Oligofructans	Hydroxypropylmethyl cellulose	
Galacto-oligosaccharides	Indigestible ('resistant') starches	
Gums		
Mucilages		
Pectins		

Plant cells contain primary cell walls and some grow thick secondary cell wall layers within the primary walls (Figure 2.1 and Table 2.2). The primary growth consists of the elongation of cell walls within chemical fractions such as polysaccharides (cellulose, xylans, pectins), protein matrix and phenolic acids (ferulic acid) are deposited (Jung & Allen, 1995). During the thickening of the secondary wall, components such as xylan, pectin and ferulic acid are less deposited in the wall in favour of lignocellulosic components. Cellulose is therefore structured into a high ordered microfibril of little variation between plants (Knudsen, 2001) and lignin is highly deposited (Jung & Allen, 1995; Jung, 1997).

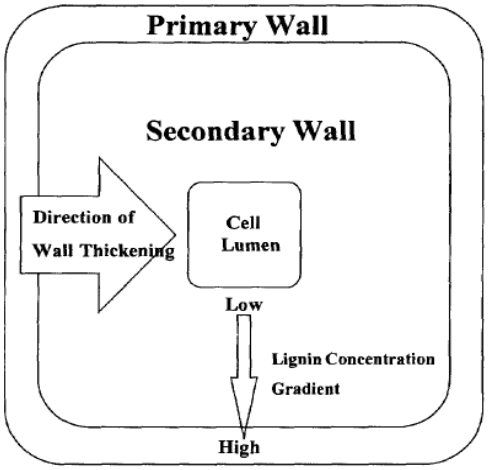


Figure 2.1 Schematic representation of a plant and wall development (Source: Jung & Allen, 1995).

As the plant tissues grow, lignin encrusts the cellulose microfibril and hemicellulose. This affects the structure of hemicellulose because of its high concentration in the primary wall (Jung & Allen, 1995; Knudsen, 2001; Graminha *et al*., 2008). The lignification transforms the overall plant cell walls in a structured and rigid barrier to prevent any physical and biochemical damages within the plant (Buxton & Redfearn, 1997; Baurhoo *et al*, 2003). This may explain why rumen micro-organisms act through the inside out digestion while digesting matured plant cell walls (Jung & Allen, 1995).

Table 2.2 Compositions of primary and secondary wall regions of mature, lignified cells in grass and legumes (Source: Allen & Jung, 1995).

Wall polymer components				
Cell wall region	polysaccharides	lignin	Phenolic acids	protein
Middle lamella/Primary wall				
Grasses	Cellulose, glucuronarabinoxylans, mixed linkage ß-glucans, heteroglucans, pectic polysaccharides (minor)	Guaiacyl (major), syringyl (minor), *p*-hydroxyphenyl (middle lamella only)	Ferulic acid esters and ethers, *p*-coumaric acid esters (minor)	Proteins with low or no hydroxyproline, extension (minor)
Legumes	pectic polysaccharides, Cellulose, heteroglucans, heteroxylans (minor)	Guaiacyl (major), syringyl (minor)	Ferulic acid esters and ethers (minor), *p*-coumaric acid esters (minor)	Extensins, other proteins
Secondary wall				
Grasses	Cellulose, glucuronarabinoxylans, heteroglucans, mixed linkage ß-glucans (minor)	Syringyl (major), guaiacyl (minor)	*p*-coumaric acid esters and ethers	None
Legumes	Cellulose, 4-*O*-methyl-glucururonxylans, glucomannans (minor)	Syringyl (major), guaiacyl (minor	*p*-coumaric acid esters and ethers	None

The physical location and chemical concentration of fibrous components within the plant cells (Table 2.2) influence the physical-chemical property of plant forages and therefore affect their dry matter content and digestibility (Buxton & Redfearn, 1997). The composition of cell wall varies largely between plant species, tissues within the plant and also between different stages of growth (Fisher *et al*., 1995; McDougall *et al*., 1996) with cellulose, hemicellulose and lignin being the major components (Graminha *et al*., 2008). Due to these components, the structural limitation to cell wall digestion at the morphological level is caused by the lignified and indigestible primary wall (Wilson & Hatfield, 1997).

2. Digestion of forage in ruminant animals

The digestion of plant cell walls is sustained by the symbiosis between the host animal and microbes in the rumen. The rumen of the animal provides the required anaerobic condition that rapidly allows micro-organisms to colonize and digest the plant cell walls via their fibrolytic enzyme secretion (Krause *et al*., 2003). Major end-products from the microbial fermentation are made available in return to the animal host (Weimer, 1998; Krause *et al*., 2003). These major end products are fatty acids (VFA; acetic, propionic and butyric acid), microbial protein synthesis (MPS), carbon dioxide (CO_2) and methane (CH_4). The VFA are absorbed through the rumen wall and constitute the major metabolic fuel for mucosal tissue and for the host animal. The MPS is the main source of protein and amino acids when digested into the small intestine (McDonald *et al*., 2002). According to NRC (2001), absorbed VFA may account up to 75 to 80% of the digestible energy requirement of the animal host, while MPS leaving the rumen may represent about 64% of metabolizable protein absorbed in its small intestine.

The outer layers of epicuticular waxes, cuticle and pectin constitute the potential and natural mechanisms of plant defence against the dehydration and the penetration of phytopathogens. In addition, the cuticular layers of grasses, legumes and cereal grains also act as a potent barrier to microbial penetration to plant cell walls in the rumen (Selinger *et al*., 1996). These barriers altogether limit the microbial attachment to plant particles and therefore the ruminal fermentation. Penetration of the feed particles by microbes normally occurs at stomata and lenticels or through any mechanical disruption (chopping, grinding and/or chewing). The microbial digestion necessarily starts from inside out (Varga & Kolver, 1997). The degree of microbial colonization and their specific mode of attachment differ between species in the rumen. The adherence is prerequisite to effective fibre digestion (Russell & Hespell, 1981). However a natural ecologically stable microbial population and its adaptation to available substrate are required in the rumen (McAllister *et al*., 1994). The microbial attachment happens in different ways, from specific mechanisms requiring binding proteins and receptors to non-specific mechanisms that require physico-chemical forces such as Van-der Waals forces (McAllister *et al*., 1994).

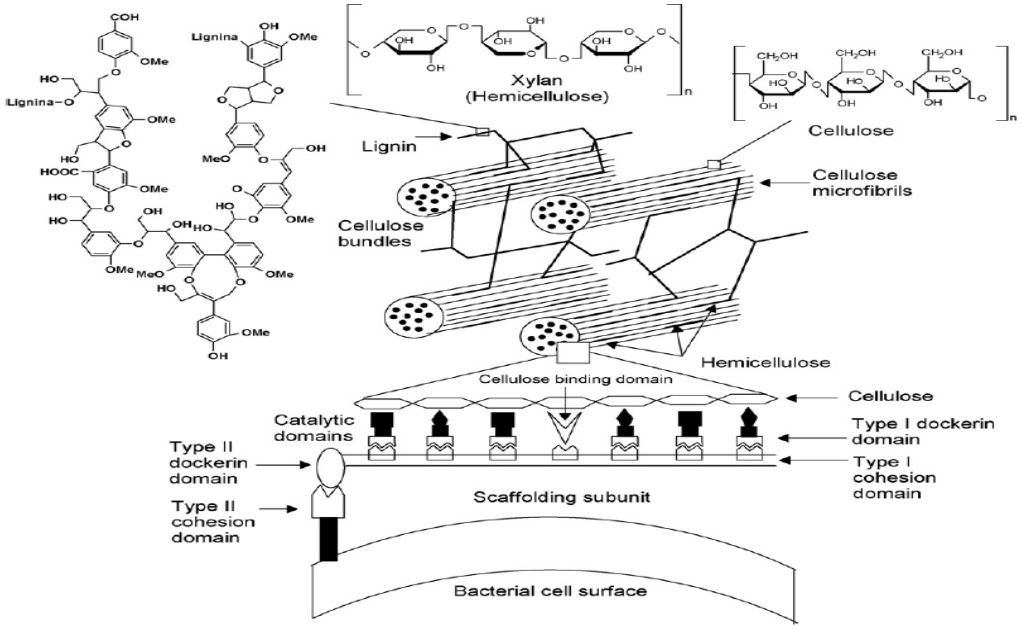


Figure 2.2 Idealized representation of fibre and its component cellulose, microfibrils, hemicellulose, and lignin that are degraded via the bacteria cellulosome complex (Source: Graminha *et al*., 2008).

The fibrolytic bacteria *F. succinogenes* (formerly *Bacteroides succinogenes*), R. *flavefaciens* and *R. albus* are generally considered to be primarily responsible for the degradation of plant cell walls in the rumen (Weimer, 1996). Figure 2.2 shows the bacterial strategies to digest cell wall components which involve a secretion of fibrolytic enzymes with high specific activities and the protein-bound adhesion by means of an extracellular glycocalyx coat and possibly by protuberances (known as cellulosomes) on the substrate (Weimer, 1996; Varga & Kolver, 1997). Furthermore, the strong adhesion as organized biofilm of bacteria to fibrous components shows advantages in digestive processes. Firstly, the cellulolytic enzymes are concentrated on the substrate excluding other microbes and their enzymes from the site of hydrolysis. This allows the rumen cellulolytic bacteria to have first access to the products of cellulose hydrolysis. Secondly, stable biofilm

communities are formed. These are resistant to detachment (McAllister *et al*., 1994) and doing so, microbes are structurally protected from a range of attacks. These attacks include antibodies, antimicrobial agents, bacteriophage, rumen proteases, predation and lysis of microbes (Weimer, 1996; Edwards *et al*., 2008).

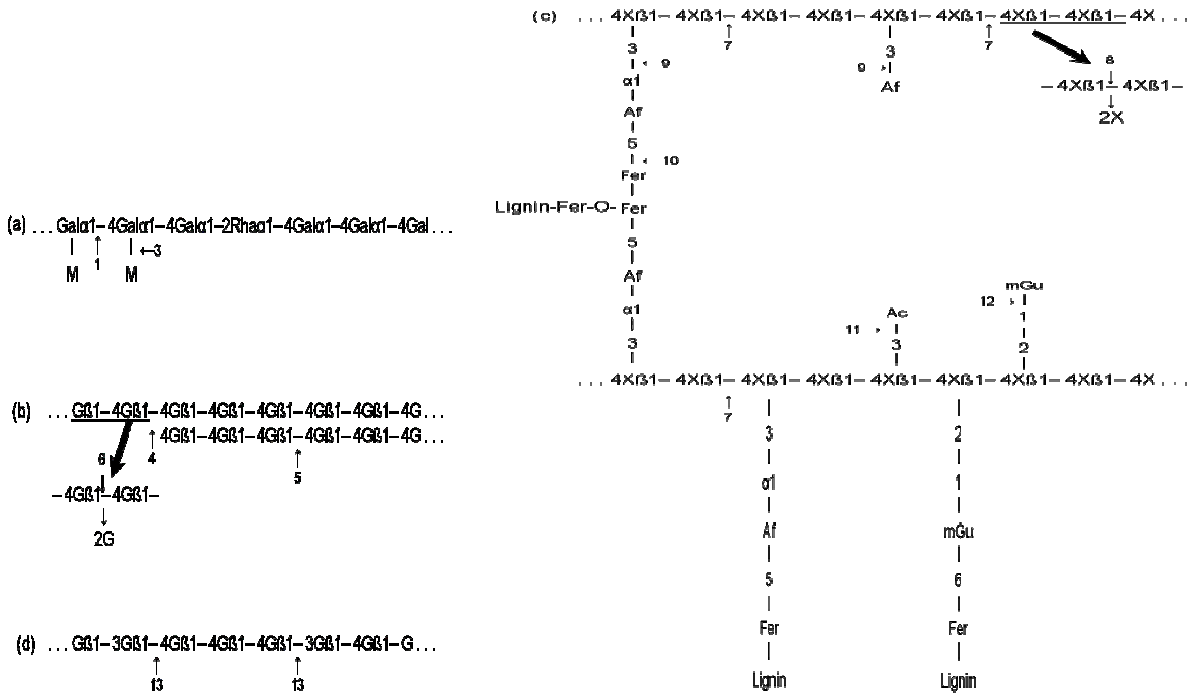


Figure 2.3 Illustration of enzymatic degradation of major chemical bonds found in the plant cell walls of grasses, legumes and cereal grains [(a) pectin, (b) cellulose, (c) hemicellulose and (d) barley-α-glucan] and enzyme cleavage sites (1 – pectin lyase, 2 – polygalacturonase, 3 – pectin methylesterase, 4 – cellobiohydrolase, 5 – endoglucanase, 6 – cellobiase, 7 – endoxylanase, 8 – xylosidase, 9 – arabinofuranosidase, 10 – feruloyl esterase, 11 – acetylxylan esterase, 12 – α-glucuronidase, 13 – mixed linkage α-glucanase). Symbols: Ac, Acetic acid; Af, Arabinose; Fer, Ferulic acid; G, Glucose; Gal, galacturonic acid; M, methyl ester; mGu, 4-O-methylglucuronic acid; Rha, Rhamnose; X, Xylose (Modified from Selinger *et al*., 1996).

Compared with bacteria, the role of the fungi and protozoa is less well understood. However fungi are well known to possess the unique capacity to penetrate the cuticle at the plant surface and the cell walls of lignified tissues. In addition, fungal enzymes present a wider range of activities, enabling them to degrade resistant plant cell wall components. This makes the fungal cellulases and xylanases the most active fibrolytic enzymes described to date (Selinger *et al*., 1996). All of the major fibrolytic enzyme activities are found in the rumen protozoan population, giving them also significant ability to digest plant cell wall polymers (Selinger *et al*., 1996). McDonald *et al.* (2002) suggest that the rumen microbes work synergistically as consortia to attack and digest fibrous components. Some like the fungi penetrate, colonize and weaken the inner tissues while others follow up to ferment the spoils of the invasion. Together they secrete an array of enzymes of different activities degrading fibrous components as described in Figure 2.3.

3. Dietary fibre and its nutritional implications

Forages, the basis of ruminant feedstuffs, contain a high proportion of 35 to 70% organic matter (Romney & Gill, 2000) with cell walls being predominant (Buxton & Readfearn, 1997). However these vast renewable resources (residues from cereal crops and pasture or cut grasses from rangelands) usually are of high cell wall and low nitrogen (N) and energy contents (Romney & Gill, 2000) and of variable quality. In ruminant nutrition, carbohydrates alone represent the highest fraction of diets and are indispensable for meeting the energy requirements of animals and maintaining the rumen health. In fact, the cell wall fraction varies from 10% in corn maize with nearly 90% dry matter digestibility to about 80% in straws and tropical grasses ranging from 20 to 50% digestibility (Fisher *et al.*, 1995). Only 10 to 35% of energy intake of forage is available as net energy (Varga & Kolver, 1997) because cell wall digestion is not efficient (Krause *et al*., 2003). Forage N consists of both protein and non protein N. The crude protein content represented as rumen degradable and undegradable protein (RDP and RUP) of any forage depends on its protein characteristics and it varies in forages as reported by Minson (1990) from < 30 to > 270 g/DM kg with a mean of 142 g/kg. Forage NPN consists of oligopeptides, free amino acids, ammonium compounds and other small molecules that rapidly contribute to the ruminal ammonia pool. The rumen conversion of forage N to microbial protein is not efficient. Kingston-Smith *et al*. (2008) reported that as little as 30% of the ingested nitrogen might be retained by the animal for milk or meat production. The non assimilated nitrogen is excreted and wasted to the environment as urea or ammonia when ruminal microbes can not utilize all of the amino acids following intense protein degradation.

Depending on the composition, structure and association of components, plant cell walls can have a large physiological effect on digestibility of plant-substrates (McDougall *et al*, 1996). Dietary fibre traps energetic and protein nutrients because of its high strength and rigidity (McDougall *et al*, 1996; Baurhoo, 2008). It influences texture and palatability of the diet and promotes satiety and reduces calorie intake. Plant fibre can modulate feed intake by increased rumen fill and reduced absorption of nutrients in the small intestine (Jung & Allen, 1995). It can also increase faecal bulk and reduces transit time (McDougall *et al*., 1996) and bind minerals due to its association to oxalates, tannins and phytates (Harland, 1989). In addition, condensed tannins found in legumes are shown to depress protein degradation by either protein alteration or inhibition of microbial proteases (Broderick, 1995). All these physiological effects of the fibre fraction may adversely affect the overall nutrient bioavailability. When formulating ruminant diets, strict considerations must therefore be taken on non structural: structural ratio of carbohydrates in estimating the energy value of feeds and minimizing the antinutritional effect of fibre components in the overall digestion.

4. Metabolism of carbohydrate and protein fractions in the rumen

Ruminant animals have the ability to convert low quality feeds into high quality protein (milk and meat) and to utilize marginal areas not suitable to grow crops for human consumption. However, the conversion of fibrous forages to meat and milk is relatively inefficient as plant cell walls recovered from faeces are still fermentable

(Krause *et al*., 2003). Only 10 to 35% of energy intake is captured as net energy because 20 to 70% of lignocellulosic biomass may not be digested in the rumen (Varga & Kolver, 1997). Kingston-Smith *et al*. (2008) reported that ruminal proteolysis contributes to the inefficient conversion of plant forages to microbial protein synthesis (MPS) and subsequently animal protein. Up to 70% of the ingested N is found to be excreted in the environment as nitrogenous pollutants in form of ammonia and urea (Kingston-Smith *et al*., 2008).

During ruminal fermentation, carbohydrates are fermented and subsequently utilized for the maintenance and growth of the microbial population. The microbial fermentation generates heat and waste products which are volatile fatty acids (VFA), methane (CH_4) and carbon dioxide (CO_2) (Russell & Hespell, 1981). In addition, the ruminal fermentation hydrolyses the protein fraction to peptides and amino acids which can be deaminated to yield urea or ammonia (Kingston-Smith *et al*., 2008). Ammonia or urea can not be taken up by the animal for growth unless first assimilated by ruminal micro-organisms. When the rate of proteolysis exceeds the relative rate of carbohydrate degradation, ammonia production can exceed the capacity for it to be assimilated by the microbial population and the excess is liberated to the environment by the animal as pollutant nitrogenous waste (Kingston-Smith *et al*., 2008). The VFA represent to the host animal the major source of absorbed energy which can account approximately 80% of the energy disappearing in the rumen. This can provide 50 to 70% of the digestible energy intake in sheep and cows at maintenance levels. In lactating cows, VFA can supply 40 to 65% of the digestible energy intake (France & Dijkstra, 2005). The majority of the VFA produced in the rumen are absorbed across the rumen wall by diffusion. However, small proportions (10-20% in sheep and up to 35% in dairy cattle) reach the omasum and abomasum and are thus absorbed from these organs (France & Dijkstra, 2005). Metabolizable protein reaching the small intestine is the net result of the production of microbial mass (MPS), the bypass protein from the rumen and endogenous protein (Sniffen & Robinson, 1987). The MPS, which provides the majority of protein, can account for 50 to 80% of the total absorbable protein in the small intestine of ruminants (Bach *et al*., 2005). In addition, MPS contain both essential and non-essential amino acids (AA), which are fairly in proportions that similarly match the overall AA spectrum of proteins being deposited in the tissues of animals (Nolan & Dobos, 2005). However, the total amount of MPS flowing to the small intestine depends on the availability of nutrients and their efficiency of utilization by ruminal microbes (Bach *et al*., 2005). This stipulates that the ruminal N metabolism relies on protein degradation, which provides N sources for bacteria and MPS.

The MPS in the rumen is influenced by the composition and supply of nutrients, microbial population and ruminal conditions (Russell & Hespell, 1981). Increasing DMI results in greater substrate flow to the rumen, which may result in greater microbial growth. The increased proportion of forage in feed DM leads to an improved retention time and greater microbial growth as microbial generation time is reduced. This is due to greater saliva flow, maintained pH, improved cation exchange capacity, improved hydration (reducing lag time), improved microbial attachment and improved formation of microbial mat (Russell & Hespell, 1981; Sniffen & Robinson, 1987; Van Soest *et al*., 1991). The greater flow of saliva flow also increases liquid outflow, which has been suggested to increase microbial outflow from the rumen (McDonald *et al*., 2002). The composition of nutrients affects the microbial growth through carbohydrate-protein synchrony in the rumen.

The synchronization of nutrients in ruminant systems has been found to enhance the yield and efficiency of MPS and the optimization of nutrient utilization and subsequently improve the animal performance (Hersom, 2008). High producing ruminants such dairy cows often are fed significant amounts of cereal grains and fat in their diets. Cereal-based diets increase the ruminal fermentation and stimulate a rapid growth of starch digesting microbes (Russell & Hespell, 1981). Furthermore, there is an accumulation of lactic acid following starch digestion. This lowers the rumen pH below 6.0 (acidosis) and disrupts the microbial ecology and the DMI (McDonald *et al*., 2002; Russell *et al*., 2009). Because of energy-wastage reactions, the extent of ruminal fibre digestion and the efficiency of MPS are often decreased (Firkins, 1996, Plaizier *et al*., 2009). The amount of dietary CP and its degradability influences microbial yield. The microbial population requires ammonia and peptides as well as amino acids for growth. The low protein intake, high degradable protein and imbalanced ratio of available soluble protein to excess available non structural carbohydrates limit the microbial growth (Sniffen & Robinson, 1987). Other factors such as protozoa preying upon bacteria, microbial death and lysis within the rumen limit the output of metabolizable protein (Russell & Hespell, 1981; Russell *et al*., 2009). The ruminal N turnover recycles significant amounts of protein. An estimated 65-85% of protozoa are reported to be recycled within normal rumen conditions (Firkins, 1996). In addition, the N turnover can be accentuated with nutritional imbalances of nutrients as a result of an asynchronous nutrient supply which impair the total density, numbers of species and viability of micro-organisms (Firkins, 1996). This shows that energy is consumed inefficiently for the resynthesis of proteins, nucleic acids and other polymers in the rumen. Feeding managements can also optimize the growth and yield of MPS and the outflow of undigested feed as a result of a continuous input of balanced nutrients. Strategies may include the frequency of feeding and nutrient delivery, the form in which the nutrients are supplied and supplement types and the attention to the balance of energy to protein ratio in the diet (Hersom, 2008). These strategies may maintain the ideal ruminal pH through increased saliva flow and stabilize fermentation rate to optimize the microbial yield (Sniffen & Robinson, 1987).

5. Limitations to plant fibre digestion

A number of factors, acting independently and / or in concert depress fibre digestion in the rumen. These are: 1) physical and chemical organization of the plant components controlling microbial attachment; 2) nature of population densities and specifity of microbes, that determine interactions between microbes in the rumen, the type and array of secreted fibrolytic enzymes and the degree of colonization and mode of attachment of each microbe specie; 3) microbial factors controlling attachment and hydrolysis by fibrolytic enzymes of adherent microbes; 4) animal factors regulating nutrient supplies through mastication, salivation and kinetics of ruminal digestion (Varga & Kolver, 1997; McDonald *et al*., 2002).

One of the major differences in fibre degradation among plant species is between grasses and legumes (Buxton & Readfearn, 1997). Waxes, cell wall structure and content, cuticle covering plants and silica regulate the access of microbes and their enzymes to inner tissues (McAllister *et al*., 1994; Varga & Kolver, 1997). Legumes are typically more digestible than grasses at respectively 40 to 50% for legumous fibre and

60 to 70% for grass fibre although grasses were found to have great NDF digestibility than legumes (Oba & Allen, 1999). Buxton & Readfearn (1997) speculated that less fibre rather than highly digestible fibre of legumes was definitely the reason. The NDF filling in the rumen might be less for legumes in contrast to grasses, but the NDF of grasses has greater particle fragility and shorter retention time (Oba & Allen, 1999). Compared to forages, cereal grains have a thick, multilayered pericarp surrounding the germ and endosperm. In addition to the pericarp, oat and barley grains also are surrounded by a fibrous husk and protein matrix. These structures are extremely resistant to microbial digestion (McAllister & Cheng, 1996).

Table 2.3 Summary of plant tissues and their relative digestibility (Source: Buxton & Readfearn, 1997).

Tissue	Function	Digestibility	Comments
Mesophyll	Contain chloroplasts	High	Thin wall, no lignin. Loosely arranged in legumes and C3 grasses.
Parenchyma	Metabolic	Moderate to high	In midrib of grass and main vein of legume leaves, leaf sheath, and stem of grasses, and petiole and stem of legumes. Highly digestible when immature.
Collenchyma	Structural	Moderate to high	In legume leaves and stems. Thick wall, not lignified.
Parenchyma bundle sheath	Contain chloroplasts	Moderate to high	Surrounds vascular tissue in C4 leaf blades. Wall moderately thick and weakly lignified.
Phloem fibre	Structural	Moderate	In legume petioles and stems. Often does not lignify.
Epidermis	Dermal	Low to high	Outer wall thickened, lignified, and covered with cuticle and waxy layer.
Vascular tissue	Vascular	None to moderate	Comprises phloem and xylem. Major contributor to indigestible fraction.
Sclerenchyma	Structural	None to low	Up to 1200 mm long and 5-20 mm in diameter, thick, lignified wall.

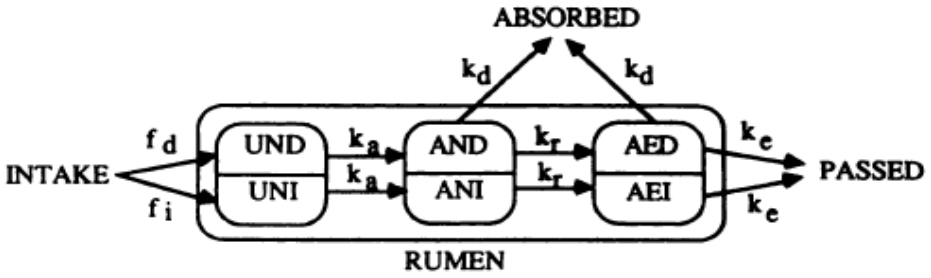
Table 2.4 Nutritive constituents of forage and limitations to their utilization by ruminants (Source: Fisher *et al*., 1995).

Component	Availability	Factors limiting utilization
Cellular contents		
Soluble carbohydrates	100%	Intake
Starch	>90%	Intake and passage rate
Organic acids	100%	Intake and toxicity
Protein	>90%	Fermentation and loss as ammonia
Pectin	>98%	Intake and passage rate
Triglycerides and Glycolipids	>90%	Intake and passage rate
Plant cell wall		
Cellulose	Variable	Lignification, cutinisation and silicification
Hemicellulose	Variable	Lignification, cutinisation and silicification
Lignin, cutin, and silica	Indigestible	Not degradable
Tannins and polyphenols	Possibly limited	Generally not degraded

The organization of plant components (Table 2.3) determines the chewing activity and thus the particle size. The particle size regulates the surface area exposed to microbes (Buxton & Readfearn, 1997), the microbial attachment and the activity of their hydrolytic enzymes (Varga & Kolver, 1997). Lignin acts as physical barrier

to microbial access at first. Then together with other polysaccharides, they act as physical and structural barriers because lignin cross-links with them in primary wall of thick walled cells by ferulate bridges (Buxton & Readfearn, 1997). Therefore many cells can be digested only from the interior of the cell (Fisher *et al*., 1995) as shown in Table 2.4.

The microbial activity in rumen is determined by many factors. These influence the population densities of predominant species of fibre digesting microbes and the nature of enzymatic activity of fibrolytic microbes on the plant cell walls. Allen & Mertens (1988) have grouped them as: (a) diet related factors: microbial activity due to the concentration of limiting substrate and diet composition (chemical composition and structure of fibre, particle size and surface area, energy and N contents, phenolic content), etc., and (b) ruminal related factors: this defines the dilution rate of the rumen due to passage rate, predation of bacteria by protozoa and other biological factors (substrate affinity, catabolite regulatory mechanisms, maximum growth rates and maintenance requirements) as well as physical-chemical factors (pH, oxidation-reduction potential, temperature, osmotic pressure, hydrostatic pressure, surface tension and viscosity). All these factors determine the rate of attachment and number of available attachment sites on the substrate, the mass of fibre digesting microbes in the rumen, the species composition of the microbial population and the ability of the different species to attach to and colonize plant cell walls (Allen & Mertens, 1988). However, pH seems to be a determinant factor of the type of ruminal fermentation that occurs and it itself set significantly by the rumen digestion (Plaizier *et al*., 2009). The growth rates of fibrolytic microbes are optimal at rumen pH 6.2 to 6.8 and the rumen pH below 6.2 compromises fibre digestion. When feeding more grains and less forage, less buffering agents (sodium bicarbonate) is produced because of low chewing and rumination activities. Besides, high production of organic acids such as VFA and lactic acid occurs in the rumen. These changes may induce a pH depression in the rumen (e.i. < 5.6 for > 3 hour per day) which can result in a decrease of number of cellulolytic microbes and subsequently in fibre digestion (Plaizier *et al*., 2009).



$$\text{Digestibility} = \frac{d\text{ABSORBED}/dt}{d\text{INTAKE}/dt} = \frac{f_d k_d}{k_d + k_r}\left(1 + \frac{k_r}{k_d + k_e}\right)$$

Figure 2.4 Model of fibre disappearance incorporating a lag phase with particles unavailable (U) and available (A) for attachment and passage. Non escapable (N) and escapable (E) as well as potentially digestible (D) and indigestible (I) fibre fractions are included. Fibre fractions and rates are represented as follows: digestible fibre as a fraction of intake (fd), indigestible fibre as a fraction of intake (fi), fractional rate of availability (ka), fractional rate of digestion (kd), fractional rate of escape (ke) and fractional rate of release from the non escapable fraction to the escapable fraction (kr) (Source: Allen & Mertens, 1988).

The rate of digestion and passage are regarded as kinetic constraints to ruminal digestion of plant cell walls. Therefore any animal and feed factors influencing the indigestible fibre fraction or acting on one of these two constraints influence the digestion in the rumen (Firkins *et al*., 1998). Allen & Mertens (1988) defined a mathematical model to evaluate these constraints on fibre digestion by rumen microbes as described in Figure 2.4. Potentially digestible fibre leaves the rumen either by enzymatic digestion or by passage to the lower tract as shown in Figure 2.4. This equation reveals that fibre digestion is described as occurring from two sequential pools. The digestibility is directly proportional to the fraction of fibre that is potentially digestible and the rate of fibre digestion, and inversely related to the rate of release of particles from the non escapable to the escapable fibre pool and the rate of escape. Following evidence from this model has shown that digestibility decreases as retention time (RT=1/kr) decreases. Both the rate of change in functional specific gravity of particles and the rate of particle size breakdown affect the rate of particle release (Allen & Mertens, 1988).

Feed factors have been also found to have effects on fibre digestion and its passage in the rumen. Firkins *et al*. (1998) discussed the effects of the composition and structure of dietary fibre and particle size on the ruminal digestion. These authors reported that the characteristics and size of fibrous components determine the structural integrity of the substrate allowing hydration and fragility of particles and the gas leakage from them. As the digestible material in particles is depleted, a low amount of fermentative gases is trapped. This allows high functional specific gravity and more floating toward the reticulo omasal orifice. Grant (1997) discussed that the fibre content and their particle sizes of fibrous components can influence the likelihood of particle escape. This is because the cell wall fraction determines the rate of rumination, chewing efficiency, microbial activity and cell wall fragility (Figure 2.5).

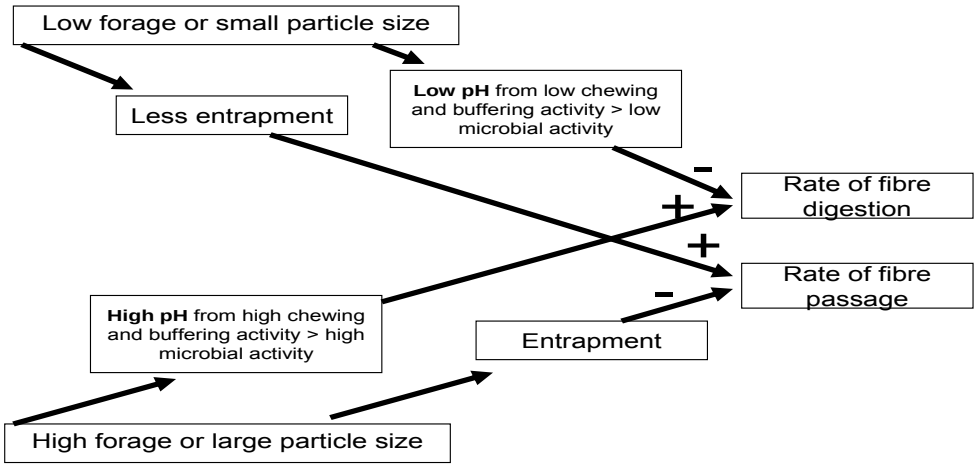


Figure 2.5 Potential interactions among forage level and particle size on kinetic digestion. (Modified from Grant, 1997).

This figure illustrates that the low amount of dietary forage increases the passage rate and limits the fibre digestion when diets with low dietary fibre or small particle size are fed instead of high forage diet. Therefore,

dietary fibre content and particle size must be adequate to stimulate rumination, avoid low rumen pH and entrap small feed particles (Grant, 1997). Animal and environmental factors can also influence the kinetic digestion. For instance, the ruminal fill and the retention time are reduced during late pregnancy. The increased demand of nutrients during lactation (early lactation or somatotropin injection) increases dry matter intake (DMI), whereas the excessive body condition loss or high environmental temperature decrease DMI. Differences among animals shown in chewing behaviours can also influence the digesta contraction in the gut and therefore affect the digestion of fibrous components and their passage rate (Firkins *et al*., 1998).

B. Fibrolytic feed enzymes in ruminant systems

Research on exogenous fibrolytic enzymes (EFE) began in early 1950, based on their potential to convert lignocellulose to glucose and other soluble sugars. These lignocellulose components are the most abundant and renewable source of energy on earth, but slowly degradable. Since their production became easy and economic in early 1980, as a result of advances in fermentation technology and biotechnology, EFE revealed their biotechnological potential in various industries (Bhat, 2000). These include food, brewery and wine, animal feed, textile and laundry, pulp and paper, agriculture as well as in research and development. In animal nutrition, the use of feed enzymes showed potential to overcome antinutritional factors (ANF) and enhance efficiency with which animals utilize the raw materials (Beauchemin *et al*., 2003). Roughages and agro industrial residues are the backbone of worldwide ruminant production. These fibrous feedstuffs, with addition to soybean and other dietary protein sources, contain some ANF which limit the efficient conversion to meat and milk. Often the limiting cause when formulating forage-based rations is the ability of ruminant to digest and absorb different nutrients of the raw material feeds, particularly plant cell walls. Van Soest (1994) reported that less than 65% of the potential nutritional value of plant cell walls is still not degraded in the rumen at the end of the digestive processes. This inefficiency of nutrient utilization can result in an increase of the diet quantity needed to maintain required levels of animal performance. This can subsequently increase the feeding cost and also the environmental pollution due to increased waste (Sheppy, 2001).

1. Biotechnology of EFE in animal feed

The Attempt to improve ruminal fibre digestion is an on-going research focus area. With 40 to 70% cell walls contained in forage dry matter (DM), several methods have been developed to optimize feed conversion. These strategies include plant breeding and management for improved digestibility (Casler & Vogel, 1999) and the increase of utilization by physical, chemical and/or microbial actions (McDonald *et al*, 2002). The EFE have shown promise at hydrolyzing plant cell walls (Bhat & Hazlewood, 2001) and revealed new opportunities to improve feed utilization in animal nutrition (Sheppy, 2001). For a more in-depth discussion of biotechnological ways to improve plant cell wall digestion in the rumen, see the review by Krause *et al*. (2003). In animal nutrition, EFE are now recognized as feed additives for their potential depolymerisation of fibrous components (Krause *et al*., 1998; Bhat & Hazlewood, 2001). The EFE, like other feed enzymes, are of natural origin and non-toxic. They are mostly commercial products of microbial fermentation of *Trichoderma*

and *Aspergillus* on safe, simple and inexpensive solid agricultural and agro industrial residues (Bhat, 2000; Graminha *et al*., 2008). These organisms are generally recognized as safe and are therefore non toxic, non pathogenic and do not produce antibiotics (Headon & Walsh, 1994). These enzymes are often used at low concentrations (Dawson & Tricarico, 1999) and are easy to apply to feed. The addition of EFE can be done during feed processing, on processed feed in storage and/or on feedstuffs in feeder bins before feeding (Pariza & Cook, 2010). These enzymes consist of mainly cellulases, xylanases and other minor enzyme complexes (Table 2.5); together they act to hydrolyse lignocellulosic materials.

The primary objective of using feed enzymes is to enhance availability of nutrients that are locked within cell wall components. Some nutrients are not as accessible to the own digestive enzymes of the animal, others are bound up in a chemical form that the animal is unable to digest them (Sheppy, 2001). The addition of enzymes is therefore to break down the anti-nutritional factors. The EFE subsequently decrease the variability in nutrient availability from feed ingredients and also supplement the digestive enzymes of the animal. Thus, enzymes can be strategically utilized to enhance the uniformity of animal performance (e.i. daily growth rate, egg production or milk production) from such intrinsically variable feed ingredients (Pariza & Cook, 2010). Improving diet utilization with EFE can enhance overall production efficiency, reduce cost of animal protein production and reduce the environmental impact of animal agriculture (Sheppy, 2001; Pariza & Cook, 2010).

Table 2.5 Role of EFE in animal feed biotechnology (Source: Bhat, 2000).

Enzyme	Function	Application	References*
Cellulases and hemicellulases	Partial hydrolysis of lignocellulosic materials; dehulling of cereal grains; hydrolysis of ß-glucans; decrease in intestinal viscosity; better emulsification and flexibility of feed materials	Improvement in the nutritional quality of animal feed and thus the performance of ruminants and monogastrics	Beauchemin *et al*., 1995; Chesson, 1987; Cowan, 1996; Galante *et al*., 1998b; Graham & Balnave, 1995; Lewis et *al*., 1996
ß-Glucanase and xylanase	Hydrolysis of cereal ß-glucans and arabinoxylans, decrease in intestinal viscosity and release of nutrients from grains	Improvement in the feed digestion and absorption, weight gain by broiler chickens and hens	Bedford & Classen, 1992; Chesson, 1987; Galante *et al*., 1998b; Walsh *et al*., 1993
Hemicellulase with high xylanase actvity	Increase the nutritive quality of pig feeds	Reduction in the cost of pig feeds and the use of less expensive feeds for pigs	Chesson, 1987; Galante *et al*., 1998b; Graham *et al*., 1998; Thomke *et al*., 1980
Cellulases, hemicellulases and pectinases	Partial hydrolysis of plant cell wall during silage and fodder preservation; expression of preferred genes in ruminant and monogastric animals for high feed conversion efficiency	Production and preservation of high quality fodder for ruminants; improving the quality of grass silage; production of transgenic animals	Ali *et al*., 1995; Hall *et al*., 1993; Selmer-Olsen *et al*., 1993

* References as cited by Bhat (2000)

2. Exogenous fibrolytic enzymes (EFE) and performance responses in ruminant systems

The forage-based diet of ruminants, which contains cellulose, hemicellulose, pectin and lignin, is more complex than the cereal-based diet of poultry and pigs. The presence of hydrophobic cuticle, lignin and its close association with cell wall polysaccharides, and the nature of lignocellulose with forage feedstuffs prevent the efficient utilization of fibre in the rumen. The use of EFE as feed additives in the ruminant nutrition is therefore done with the purpose of improving the nutritive quality of forage in order to increase rumen degradation of plant cell walls (Eun & Beauchemin, 2007). In this regard, cellulases and xylanases are respectively two major fibrolytic enzyme groups (Bhat & Hazlewood, 2001). These are specified to break ß1-4 linkages joining sugar molecules of cellulose and xylans found in plant cell wall components (Dawson & Tricarico, 1999; Beauchemin *et al*., 2003). However, the success of these EFE in ruminant diet in order to guarantee success depends on: (1) their stability on the feed (during and after processing) and in the rumen; (2) their ability to hydrolyse plant cell wall polysaccharides; and (3) the ability of the animals to use the reaction products efficiently (Bhat, 2000).

Several investigations with EFE have made mention of the improvement of microbial activities in the rumen with positive enhancement on the animal performance (Lewis *et al*., 1999; Rode *et al*., 1999). Despite the increase of feed digestibility and ruminant performance traits, the relationship between improvement in forage utilization and enzymatic activities of EFE is not yet fully explained (Eun *et al*., 2007). In addition, EFE in ruminant systems are of variable results (Beauchemin *et al*., 2003; Colombatto *et al*., 2003). This makes their biological response difficult to predict. Some studies have shown substantial improvements in feed digestibility and animal performance (Yang *et al*., 1999 ;Cruywagen & Goosen 2004; Bala *et al*., 2009), while others reported either negative effects or none at all (Vicini *et al*., 2003; Bowman *et al*., 2003; Baloyi, 2008).

Bala *et al*. (2009) found a significant improvement of digestibility and total carbohydrates when EFE containing cellulase and xylanase activities were applied on concentrate supplement of lactating goat. Similary, Nowak *et al*. (2003) reported that EFE increased DM, NDF and acid detergent fibre (ADF) disappearances of wheat straw and TMR during the initial phase of digestion. In contrast, Lewis *et al.* (1996) found no effects of EFE during the initial phase of digestion, but EFE improved DM and NDF disappearance after 32, 40 and 96 hours of incubation. Colombatto *et al*. (2003) and Eun *et al.* (2007) tested different EFE with xylanase and endoglucanase activities to improve forage digestion using a gas production (GP) system. It has been demonstrated that EFE increased the organic matter degradation of lucerne hay of both leaves and stems after 12 hours of incubation (Colombatto *et al*., 2003). All enzyme treatments increased the extent of degradation (96 hours of incubation) in the leaf fractions, but only EFE with endoglucanase activity increased final OMD in the stems. Eun *et al.* (2007) showed that EFE increased GP and degradation of lucerne hay and corn silage at the optimum dose rate (1.4 mg EFE/g DM) with improvements in NDF degradability up to 20.6% and 60.3%, respectively.

Beauchemin *et al*. (1995) reported that the addition of commercial EFE preparations containing cellulases and xylanases to a forage-based diet increased the live weight gain of cattle by 35%. Balci *et al.* (2007)

reported that EFE with cellulases and xylanases improved the total live weight gain, average daily gain (ADG) and total feed conversion rate. These were respectively affected as follows: 69.0 kg, 986.0 g, and 11.42% for control treatment against 88.9 kg, 1270.0 g, and 8.94% for enzyme treatment when fattening steers in 80 days. Bala *et al*. (2009) also found that adding EFE to concentrate supplement in the last quarter of lactation improved body weight and milk production of goats. Similarly, an increase of 5 to 10% in milk yield has been reported with dairy cows maintained on forage treated with commercial EFE (Lewis *et al*., 1999; Rode *et al*., 1999; Yang *et al*., 1999). In contrast, no significant effects either in body weight or milk yield were observed in other studies (Vicini *et al*., 2003).

Goosen (2005) screened many different EFE to improve the degradation of wheat straw using the GP system. The author reported that strain Abo 374 increased the cumulative GP by > 10% at 18 hours. Consistent with this, Cruywagen & Goosen (2005) reported that the medium dose rate (5 ml supernatant/kg of wheat straw) of the same strain increased growth rates and feed conversion ratios by 7.13 kg and 0.16 in growing lambs, compared to 5.41 kg and 0.12 of control treatment at 6 weeks. An increased animal performance has also been shown with the same enzyme on both high and low forage-based diets in another study done by Cruywagen & Van Zyl (2008). In contrast, Baloyi (2008) found no effects on GP and *in vitro* DM and NDF digestion when the same enzyme product was added to forage hays and mixed feed substrates.

Thus, the use of EFE to improve fibre digestion in ruminant systems is afflicted by the variation of results. This limits the biological prediction and therefore the overall success of EFE in ruminant systems. Inconsistent and variable responses were found to be caused by the differences in enzymes (key activities, level of supplementation, methods of application, etc.), substrates (enzyme-feed specificity, type of diet) and the energy balance of the test animals (Beauchemin *et al*., 2003). Hence, considerable basic and applied research efforts, together with improved enzyme formulations, are still needed to limit variations on EFE responses in ruminant systems, enhance ruminal fibre digestion and consequently improve the animal performance.

3. Possible mode of action of EFE in ruminant systems

The mode of actions of EFE in ruminant systems is not conclusive (Beauchemin *et al*., 2004). This is due to the lack of understanding the relationship between enzymatic activities and the improvement in forage utilization (Eun *et al*., 2007). Previous works on this topic showed that EFE can act to improve feed utilization in ruminants either through their effects on the feed before consumption or through their enhancement of digestion in the rumen and/or in the post-ruminal digestive tract (McAllister *et al*., 2001).

The EFE are most effective when applied in liquid form onto dry feed prior to ingestion (Kung *et al*., 2000; Beauchemin *et al*., 2003). This may partially digest feed or weaken cell wall barriers that limit microbial digestion in the rumen. The direct action of EFE before feed consumption can cause a release of reducing

sugars (Hristov *et al*., 1996) arising from partial solubilisation of cell wall components (Krause *et al*., 1998). This may therefore increase available carbohydrates in the rumen required to shorten the lag time needed for microbial colonization and also enhance the rapid microbial attachment and growth (Forsberg *et al*., 2000). The alteration of feed structure, due to the partial solubilisation of cell wall before feeding, is more likely to increase feed degradation in the rumen (Beauchemin *et al*., 2004). Another important advantage for treating feed with EFE prior to ingestion is the improvement of the enzyme binding to feed particles, in contrast to its direct infusion in the rumen. This was thereby reported to increase the resistance of EFE to proteolysis in the rumen (Morgavi *et al*., 2001; Beauchemin *et al*., 2003).

In the rumen, EFE may hydrolyse feed directly or work synergistically with ruminal microbes to enhance feed digestion (McAllister *et al*., 2001). Wallace *et al*. (2001) studied the stability of EFE in the rumen fluid. Their findings revealed that an EFE addition to the diet at 1.5 mg/g increased xylanase (measured using oat spelt xylan) activity by 5% and cellulase (measured using carboxymethyl cellulose) activity by 15%. Consistent with this, Hristov *et al*. (1998) demonstrated that applying EFE at 12 mg/g can increase xylanase and cellulase activities respectively by 32% and 11% in the rumen. These two studies elucidated that EFE were actively stable to continue hydrolysing feed in the rumen fluid. Evidences of the stability of EFE in the rumen demonstrated the substantial synergism between EFE and ruminal enzymes such that the net combined hydrolytic activity in the rumen is much higher than estimated from single sources (Beauchemin *et al*., 2004). This positive synergy was reported as a result of an increased *in vitro* GP, total VFA, true degradability of substrate DM and a decreased methane production (Giraldo *et al*., 2008a). Morgavi *et al*. (2000) speculated that the synergy is likely a significant mechanism by which enzyme additives improve feed digestion. In sub-rumen conditions (pH > 5.9) resulted from using high fermentable diet, EFE effectiveness was considered to be reduced compared to its effectiveness at higher rumen pH conditions (Beauchemin *et al*., 2004). Yang *et al*. (2002) revealed that the effects of EFE rather than enhanced microbial activity improved ruminal fibre digestion during sub-optimal ruminal conditions.

Another evidence of EFE application in ruminant systems is the indirectly increase of attachment and numbers of cellobiose- and glucose- utilizing bacteria in the rumen (Nsereko *et al*., 2002). Similarly, Giraldo *et al*. (2008b) found that treating high-forage diet with EFE stimulated the *in vitro* numbers of microbes and enhanced the fibrolytic activity. The microbial stimulation can increase the availability of substrate as a result of an improved cell wall digestion and may accelerate the digestion of newly ingested feedstuffs (Beauchemin *et al*., 2004). This may amplify the synergy between EFE and ruminal enzymes. Furthermore, the stimulation of total microbial numbers by EFE can result in greater micro-organism biomass and would impact the supply of metabolizable protein to the small intestine (Yang *et al*., 1999). Thus, improvements in digestibility due to an increased hydrolytic activity can also attributed to an increased digestion of non-structural components in addition to an increased fibre digestion (McAllister *et al*., 2001). This may explain why EFE can be effective in high concentrate diets (Beauchemin *et al*., 2004).

In the small intestine, EFE appear to survive for a sufficient period of time with sufficient effects on substrate particles when applied to wet feeds and concentrate premix (Morgavi *et al*., 2001; Beauchemin *et al*., 2004).

This may improve nutrient absorption by hydrolyzing substrates that rapidly escape ruminal digestion. It makes possible for the remaining EFE to work synergistically with microbes in the large intestine (Beauchemin *et al*., 2004). Knowlton *et al*. (2007) observed that feeding exogenous phytases and cellulases to lactating cows improved the digestibility of diet and reduced the faecal excretion of DM, NDF, N and P fractions. This may improve the rate of decomposition of faeces and reduce therefore overall manure output in ruminant agriculture.

In conclusion, Sajjad *et al*. (2008) suggested that EFE as feed additives in ruminant systems can improve feed digestion within the rumen either by pre-treating the feed with EFE or by directly increasing the fibrolytic activity into the rumen.

C. Methods to evaluate ruminant feeds

The ruminant production systems are dependant worldwide on pasture-based diets as the main nutritional components (Wilkins, 2000). However, high levels of production and nutrient demand of ruminants such as dairy cows can not be reached to support milk production under grazing conditions as pasture-based diets have constraints that limit effective digestion (Kolver *et al*., 2003). Low pasture DM intake has been identified as a major factor limiting milk production from high-producing cows under grazing conditions (Mould, 2003). In addition, other nutritional factors such as metabolizable energy and protein affect the level of production. These have been attributed to a low supply of ME and an inefficient capture of rumen N as microbial protein (Kolver *et al*., 2003). Therefore, feed evaluation systems (Figure 2.6) attempt to estimate the capacity of a feed to sustain animal production and to supply nutrients required for a certain animal production class (Beever & Mould, 2000; Mould, 2003).

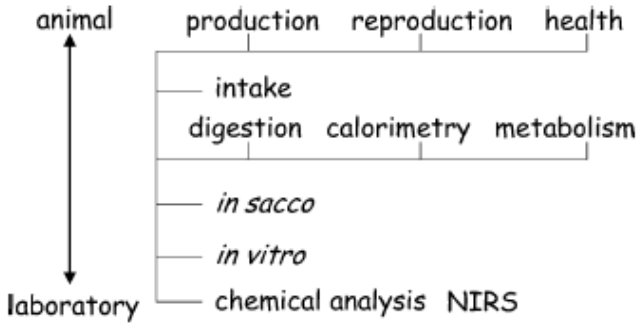


Figure 2.6 Range of feed evaluation, with NIRS: Near-infrared reflectance spectroscopy (Source: Mould, 2003).

The quality of animal feedstuffs is accurately estimated *in vivo* where animal-feed interactions are considered. The quality is thus evaluated through animal performance traits as they remain the ultimate arbitrator of nutritional value (Mould, 2003). This is measured by the estimation of intake, digestibility and efficiency of utilisation of feedstuffs in question. Of these, the variation of intake represents 60 to 90% of variation on the

available digestible energy to the animal. Forage characteristics referring to intake and digestibility are also important to measure as an index of nutritional value (Cherney, 2000). Chemical nutrients associated with intake and digestibility consist of cell wall components and protein fractions. As these settle the nutrient supply and thus the animal performance (McDonald *et al*., 2002), the routine analysis of forages should consist of a determination of these components as well as the DM and ash. Estimation of other additional components such as water-soluble carbohydrate, starch, tannins, etc. is dependant on the desired objectives of the specific research (Cherney, 2000).

The nutrient composition of feed is commonly estimated by chemical analysis (proximate analysis). This provide information about the concentrations of nutrients (DM, NDF, CP, ash) as well as the inhibitors and structures that may impact the availability of nutrients. This procedure is easy and fast. However it doest not provide sufficient informations about the true nutritive value of the feed. It is the digestive efficiency, by which a ruminant animal utilizes feed nutrients, that has a significant impact on its productivity performance and waste production (Cherney, 2000). Effects such as palatability, the impact of diet composition on digestibility or the extent to which anti-nutritive factors influence feed intake, can not be determined with laboratory analyses (Mould, 2003). As a result, various biological methods involving different procedures have been developed to evaluate feeds in ruminant systems. The *in vivo* methods involve markers and the *in sacco* method needs animals that are fitted with rumen fistula (cannula). Feed evaluation studies with respect to health, reproduction and production traits are expensive in terms of the number of animals, quantity of feed, time, labour and facilities required. As a consequence they are generally undertaken to confirm results obtained from *in vitro* and *in sacco* screening works. On the other hand, the estimations of digestion, nutrient utilisation, calorimetric and intake provide highly detailed information and they are obtained under highly controlled experimental (Mould, 2003). The *in vitro* methods utilize rumen fluid, which is obtained from fistulated animals, to estimate either digestibility or gas production (GP). Other *in vitro* methods involving commercial proteolytic enzymes, faeces or solubility in solvents and buffers are also available (Mohamed & Chauldry, 2008).

1. Proximate analysis and Van Soest analysis

The proximate analysis or Weende classification system has been in use for over a century. This includes components, namely crude protein, ether extract, crude fibre, ash and by difference, nitrogen (N)-free extracts (Figure 2.7) (Fisher *et al*., 1995). The Weende procedure is simple, repeatable and relatively cheaper, but several problems with its accuracy in the determination of components limit its use. For instance, the total carbohydrate, which is divided into crude fibre and N-free extracts, is criticized as being imprecise. In doing this, the proximate analysis stipulates that the crude fibre is formed of all dietary cellulose, hemicellulose and lignin (Cherney, 2000) whereas crude fibre has soluble and insoluble fractions both in the neutral detergent solution and acid detergent solution (Van Soest, 1982). The Van Soest method has been designed to fractionate feed dry matter in three classes: completely available, partly available due to lignification and unavailable fractions (Van Soest, 1994).

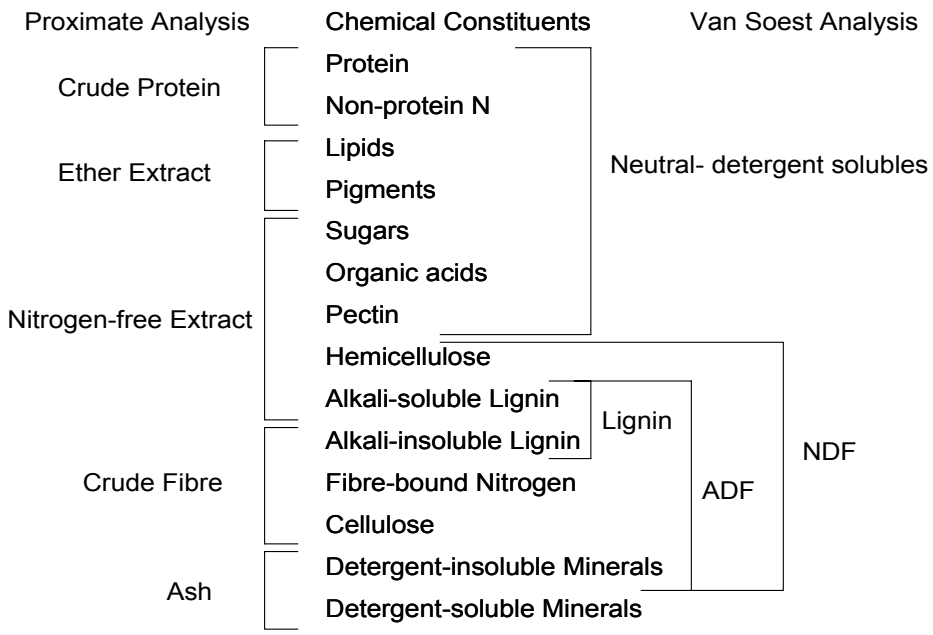


Figure 2.7 Contrast of Weende system and Van Soest system of carbohydrate analysis (modified from Fisher *et al*., 1995), with ADF as acid-detergent fibre and NDF, neutral-detergent fibre.

The extraction of forage with a neutral solution (pH 7.0) of sodium lauryl sulphate and EDTA dissolve the cell contents and the remaining is the insoluble plant cell walls (NDF). The neutral-detergent fibre (NDF) consists mainly of lignin, cellulose and hemicellulose (Figure 2.7). Minor components associated with cell walls such as protein and bound nitrogen, minerals and cuticle are also present in the NDF residue. The use of sodium sulphite anhydrous (Na_2SO_3) in the NDF solution during extraction and the heat stable α-amylase during rinsing with warm water are recommended to decrease nitrogen and starch contamination in NDF determination (Van Soest *et al*., 1991).

The acid-detergent fibre (ADF) analysis consists of an extraction of forage with an acid solution of 0.5 M sulphuric acid and cetyltrimethyl-ammonium bromide (Van Soest, 1982). ADF residue does not consist of all cell wall components, as hemicellulose is soluble in the acid-detergent solution (Fisher *et al*., 1995). It represents a fraction of NDF formed of cellulose, lignin, maillard products, acid-insoluble ash and acid-detergent-insoluble nitrogen (Cherney, 2000).

2. *In sacco* method to estimate feed degradation

Since it was first suggested by Quin *et al*. (1938), the *in sacco* technique has been recommended to estimate the utilisation of either forages or concentrates and high-protein feeds. The basement of this technique was well acknowledged since Mehrez & Ørskov (1977) studied factors causing the variability in DM and N degradability. They revealed that as long as the bags were large enough to allow free movement of substrate within, the technique could be extremely useful as a rapid guide to study the rate and extent of disappearance

of nutrients from the rumen. This technique is efficient for testing feed in the dynamic ruminal environment (i.e. pH, temperature and CO_2) and also to evaluate the degradability of DM, NDF and CP fractions in the rumen. However, the *in situ* nylon bag technique utilizes cannulated animals and the tested feed is not subjected to mastication and rumination as it would be in the *in vivo* method. Compared to the *in vivo* method, this technique is still more reliable because it needs fewer measurements and has relatively less labour inputs. Therefore it is a cheaper technique. The fistulation of animals still limits its use in research due to its implications for animal welfare and costs. Thus, *in sacco* methods, like *in vivo* methods, can not be taken in consideration as methods for routine screening of feedstuffs (Mohamed & Chauldry, 2008)

The *in sacco* technique consists of digesting forage samples in nylon, polyester or Dacron bags in suspension in the rumen for different periods of time, following by the determination of DM and protein after washing residues with running water (McDonald *et al*., 2002). Despite its widespread use, the technique has shown different sources of errors in laboratory results as reviewed by Mohamed & Chauldry (2008) and Vanzant *et al*. (1998). These sources of variation include: bag difference (size, porosity) and characteristics of feed sample (variety, agronomic conditions and processing, sample weight in a given bag size), technique manipulations, microbial contamination to feed residues, animal variation and time (hours) of incubation used in different studies.

3. *In vitro* methods to estimate nutrient degradation

Various *in vitro* techniques have been used in the past as alternatives to the *in sacco* method. These consist of the use of rumen fluid, buffers, chemical solvents or commercial enzymes. Another technique uses the gas production (GP) system as an indirect measure of the *in vitro* digestion. The focus of discussion is on the *in vitro* methods using the rumen fluid.

In vitro techniques using rumen fluid are considered as methods for routine screening of feedstuffs due their high correlation with the *in vivo* digestibility (Holden, 1999). In addition, they are cheaper, easier and faster than the *in vivo* and *in sacco* methods. These techniques offer the possibility of analysing both the residue and the metabolites of microbial degradation. Furthermore, they allow control over various factors that alter the feed degradation (microbial, animal, environment) and provide uniform characterisation of feeds for DM and protein degradation (Mohamed & Chauldry, 2008). Although the *in vitro* techniques were developed as alternatives to the *in sacco* method to study the ruminal degradation of feeds, they are still unable to remove the need to use fistulated animals to collect rumen fluid.

All *in vitro* techniques currently in use (gas production system and ANKOM technique) are adapted from a method described by Tilley & Terry (1963). This method consists in its first stage (as in the rumen) of incubating feed sample at 39º C in rumen fluid, which is diluted with a buffer solution similar in characteristics to saliva and saturated with CO_2 to maintain anaerobic conditions. After 48 hours, the incubation is stopped and the incubation mixture filtered. The filtered residues are subsequently incubated in its second stage (as in

the lower digestive tract) for another 48 hours with pepsin-HCl to remove undegraded plant cell matter and microbial protein (Beever & Mould, 2000). The two-stage technique has still an inconvenient to use donor animals for rumen fluid. In addition, it only provides an end point measurement of digestion but not any information about the kinetic of digestion (Theodorou *et al.*, 1994). To improve the post rumen digestibility, Goering & Van Soest (1970) introduced the treatment of residues with the NDF solution

In vitro methods involving the GP system consists of the measurement of the volume of gas produced by fermenting feedstuffs using rumen fluid from fistulated ruminant and buffer solution (Menke *et al*., 1979; Krishnamoorthy *et al*., 2005). These techniques, which collect and measure gas, range from the use of calibrated syringes (Menke *et al*., 1979) and pressure transducers (Theodrorou *et al*., 1994) to computerised gas monitoring devices (Pell & Schofield, 1993). The advantage of the automated gas production system is of high accuracy and reduction of the labour input. However, this option does not allow easy manipulations of large numbers of samples and is expensive when compared to the manual method (Mohamed & Chauldry, 2008).

According to Pell & Schofield (1993), the gas is produced from both soluble and insoluble metabolic energy sources. The *in vitro* GP intends to measure the potential conversion of different nutrient fractions (monosaccharides, polysaccharides, pectin, starch, cellulose and hemicellulose) to CO_2, VFA and CH_4. Many factors as reviewed by Mohamed & Chauldry (2008) are likely to affect the accuracy of the GP technique. These include: sample characteristics, buffer composition, ratio of rumen fluid inoculum and buffer solution, prevailing pH and temperature, atmospheric pressure and stirring. Despite its poor correlation to the *in vitro true* digestibity (Getachew *et al*., 2004), the GP system is widely used due to its potential to accommodate large numbers of samples. It is also cheap, less time consuming and allows accuracy over experimental conditions than the *in vivo* trials (Getachew *et al*., 1998). High correlations between GP and NDF disappearance, R^2 = 0.99 (Pell and Schofield, 1993) or GP and DM disappearance, R^2 = 0.95 (Prasad *et al*., 1994) have been reported. Although the GP system is suitable to screen large numbers of feedstuffs or treatments by giving informations on rate and the extent of fermentation, it does not provide direct informations of both the rate and extent of feed degradation or the quantity of end products fermentation (VFA and MPS) available to the animal (Mauricio *et al*, 1999).

An ANKOM incubator and fibre apparatus developed by ANKOM® Technology Corp. (Fairport, NY, USA) were introduced to improve the estimation of *in vitro* true digestibility. The method consists of digesting forage samples into filter bags in suspension in the mixture of buffered solution and rumen fluid for different periods of time, within rotating digestive jars in an insulated incubator (DAISY^{II} incubator). Besides being highly correlated to the *in situ* method (Spanghero *et al*., 2003), the filter bag technique is efficient to determine the rate and extent of degradation of feedstuffs (Holden, 1999). In addition, it reduces labour input as the technique prevents the filtration of residues in the estimation of *in vitro* digestibility (Cherney, 2000). Furthermore large numbers of feeds, different forages, grains and mixed feeds can be incubated together in a single digestion jar. The DAISY^{II} technique is seen as a rapid and convenient tool to evaluate *in vitro* digestibility of feeds in ruminant systems.

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Aim and objectives

As reported in the previous section, the addition of exogenous fibrolytic enzymes (EFE) in ruminant diets has been shown to have a positive effect in ruminant systems. With the intake of digestible energy by ruminants being limited by cell wall degradability in the rumen, attempts to enhance cell wall digestion with biotechnological products such as EFE were re-evaluated using small ruminants. The aim of study was therefore to evaluate the effect of EFE on crude protein (CP) digestion in relation to dry matter (DM) and fibre digestion, to subsequently improve microbial protein synthesis (MPS) in a ruminant system.

Firstly, a preliminary assessment using a 24 hours gas production (GP) system was conducted with three potential EFE (Abo 374, EFE 2 and EFE 3) products and one microbial yeast preparation on four different substrates (lucerne hay, wheat straw, wheat straw treated with urea and concentrate diet) as an indication of efficacy and potential to alter fibre digestibility. After the identification and selection of the most promising EFE, a study was then conducted with the objective to evaluate the effect of two EFE on rumen protein and fibre digestion and MPS, using fistulated Döhne-Merino sheep. The main objective of this research was to determine the effect of EFE treatment of forages on CP and MPS. The specific objectives were to:

1) evaluate EFE for its impact on CP degradation and NDF digestibility in the rumen using *in vitro* techniques (*in vitro* filter bag technique and GP system);

2) determine the relationship between MPS and the cumulative GP at 48 hours of incubation;

3) to further determine the effect of the superior enzyme identified from the previous activity in a parallel *in vitro* and *in situ* disappearance study using cannulated Döhne-Merino sheep.

As EFE is known to potentially depolymerise plant call wall components, it was hypothesised that EFE has a stimulatory effect on MPS. The research reported in the following chapters of this document was conducted *in vitro* and *in situ* with the assumptions that potential EFE, identified as having a positive effect on DM and NDF digestion, would improve the degradation of CP and also enhance the MPS yield thereafter.

CHAPTER 3

General materials and methods

A study to evaluate the effects of exogenous fibrolytic enzymes (EFE) in ruminant (sheep) diets on rumen crude protein (CP) and microbial protein synthesis (MPS) in relation to fibre digestion was conducted at Stellenbosch University, South Africa (33º 55′ 12″ South, 18º 51′ 36″ East). This chapter would describe the materials and methods used throughout this study, outlining the preparation of feed samples, EFE treatment, buffered solution, collection of rumen fluid, *in vitro* procedures, etc.

1. Preparations of feed samples

The chemical compositions of the lucerne hay (*Medicago sativa*), wheat straw (*Triticum aestivum*), wheat straw treated with urea, concentrate diet and a mixed substrate of lucerne hay and wheat straw are presented in Table 3.1. These four single samples were tested using *in vitro* techniques (*in vitro* GP system and nylon bag technique) in Chapters four and five. The mixed substrate of lucerne hay and wheat straw was assessed in a parallel *in vitro* and *in situ* evaluation in Chapter six.

Table 3.1 Proximate analysis of substrates used in the assessment of EFE.

Substrates	DM (g/kg)	Ash (g/ DM kg)	OM (g/ DM kg)	NDF (g/ DM kg)	ADF (g/ DM kg)	CP (g/ DM kg)
Lucerne	873.93	72.93	927.07	306.05	228.48	157.03
Wheat straw	891.41	95.35	904.65	709.43	429.92	39.77
Wheat straw with urea	916.79	100.19	899.81	760.60	507.25	93.25
Concentrate diet	884.80	64.22	935.78	242.38	172.17	117.93
1:1 lucerne hay and wheat straw	900.55	52.76	947.24	665.18	339.54	93.76

DM: dry matter, OM: organic matter, CP: crude protein, NDF: neutral-detergent fibre, ADF: acid-detergent fibre

Substrate samples were milled through a 2 mm screen (Hammer Mill Ser. No. 372, Scientech RSA, Cape Town, RSA) and sieved for 5 to 7 minutes with a mechanical shaker (model Siemens Schuckert,J. Engelsman, Ludwigshafen, a. Rh. Germany) using a 125 µm sieve to remove dust and extremely fine particles. The sieving procedure reduces the variation of particle size within a particular sample (Tilley & Terry, 1963). Fine particles can pass through pores of nylon bags, thus influencing results by overestimation of the soluble fraction (Cruywagen, 2003). To improve quality of the low CP in wheat straw, non protein nitrogen (NPN, urea) was added in order to double the N content of wheat straw. Therefore, 2% urea relative to weight was diluted in distilled water, sprayed on wheat straw and oven-dried at 60º C for 72 hours to produce the substrate wheat straw treated with urea. The mixed substrate of lucerne hay and wheat straw was obtained from separate ground and sieved lucerne hay and wheat straw prior to mixing at a ratio of 1:1. All samples were then stored in sealed plastic boxes at 4º C until required, so as to preserve a constant chemical composition.

2. Animals and diets

Döhne-Merino cannulated sheep (Stellenbosch University animal care and use committee SU ACUC, Ethic clearance number: 2006B03005) were maintained on a standard forage-based diet [lucerne hay and wheat straw at 1:1 ratio and 0.5% urea in premix] supplemented with 300 g/day of concentrate [880 g/kg of DM, 120 g/kg of CP, 200 g/kg of NDF, 10 g/kg Ca and 8 g/kg of P]. This ration was called basal diet A. The standard diet and water were offered *ad libitum* to animals held in pens. An adaptation of ten days to the basal diet was allowed before the collection of rumen liquor for the *in vitro* evaluations.

For the latter part of the study where *in vitro* and *in situ* digestibility evaluations were conducted in parallel, four cannulated sheep were used. They were maintained on a ration, named basal diet B, consisting of a standard diet [lucerne and wheat straw at 1:1 ratio (29.85% each), 14.92% corn starch, 23.88% molasses meal and 1.5% premix] supplemented with 300 g/day of concentrate [880 g/kg of DM, 100 g/kg of CP, 250 g/kg of NDF, 15 g/kg Ca and 2 g/kg P]. The standard diet and water were offered *ad libitum*. An adaptation of ten days to the basal diet was needed before the incubation periods.

3. Treatment preparations

Four treatments consisting of three exogenous fibrolytic enzymes (EFE: Abo 374, EFE 2 and EFE 3) and one microbial yeast preparation (M-yeast) were tested. All treatments were applied 12 hours prior to incubation in order allow enzyme-substrate interaction (Beauchemin *et al*., 2003). This was to create a stable enzyme-feed complex and to start the alterations of fibre structure thereafter. The control treatment consisted of distilled water.

Abo 374 is an extracellular enzyme of a South African fungal strain, cultivated on wheat straw. Abo 374 has cellulases, xylanases and mannanases, with xylanase as the major fibrolytic activity (Cruywagen & Van Zyl., 2008). This enzyme was developed at the Department of Microbiology (Stellenbosch University). The Abo 374 treatment was prepared by weighing 0.5 g enzyme powder in 68 ml of distilled water as per recommendation of Goosen (2005). The enzyme dilution was obtained by mixing one ml of concentrate enzyme with 200 ml of distilled water. This was subsequently used as a treatment at a ratio of one ml to 0.5 g substrate for *in vitro* studies and one ml per gram of substrate for the *in situ* trial. A dose of enzyme concentrate at a ratio of five ml per kg was sprayed on the standard diet fed *ad libitum* during the *in situ* experiment.

EFE 2 (Cattle-Ase^{TM}, Loveland Industries Inc., Greeley, CO, USA) is a commercial enzyme product. This treatment was prepared by weighing 1.6 g enzyme granulate in 100 ml of distilled water. The enzyme dilution was obtained by mixing one ml of concentrate enzyme with 200 ml of distilled water. This was subsequently used as a treatment at a ratio of one ml to 0.5 g substrate for *in vitro* studies.

EFE 3 (Pentopan® Mono BG, Novozymes, Denmark) is a registered commercial enzyme produced from *Aspergillus oryzae*. It has endo-1, 4-xylanases as major enzyme activity (Pentopan data sheet, www.novozymes.com). The Pentopan treatment was prepared by mixing 2.0 g granulate enzyme in 100 ml of distilled water. The enzyme dilution was obtained by mixing one ml of concentrate enzyme with 200 ml of distilled water. This was subsequently used as a treatment at a ratio of one ml to 0.5 g substrate for *in vitro* studies.

Microbial yeast (Levucell© SC, Lallemand Animal nutrition, USA) is a direct-fed microbial product containing *Saccharomyces cerevisiae* at a ratio of 3.3 x 10^9 coli form unit (CFU) /g. This treatment was made with one g of granulate product diluted in 300 ml of distilled water. The enzyme dilution was obtained by mixing one ml of concentrate enzyme with 10 ml of distilled water. This was subsequently used as a treatment at a ratio of one ml to 0.5 g substrate for *in vitro* studies.

4. Preparation of *in vitro* medium and reducing solution

The reduced buffer solution for the *in vitro* techniques (*in vitro* GP system and ANKOM® technique) was based upon the *in vitro* rumen digestibility buffer solution. Medium was prepared as described by Goering and Van Soest (1970) with slight modification. The modification consisted of using tryptose instead of trypticase. The medium consisted of macro minerals, micro minerals, tryptose, rezasurin and distilled water (Table 3.2).

Table 3.2 Complete recipe of the reduced buffer solution used in the *in vitro* digestion.

Composition	1 L volume
Distilled water (ml)	500
Tryptose (g)	2.5
Resazurin 0.1% W/v (ml)	1.25
Macro mineral (ml)	250
Micro mineral (ml)	0.125
Buffer solution (ml)	250
Reducing solution (ml)	50

Table 3.3 summarizes the constituents of the reduced buffer solution. When gases are released during 96 hours of incubation, the buffer solution containing soduim bicarbonate content is strong enough to maintain a pH range above 6.2 for 0.40 to 0.60 g of fermented substrate (Mertens & Weimer, 1998). The reducing solution consisted of cysteine hydrochloride ($C_3H_7NO_2 \cdot HCL$), potassium hydroxide (KOH) pellets, sodium sulphide monohydrate ($Na_2S \cdot H_2O$) and distilled water. The addition of trace minerals and tryptose (a pre-digestive source of amino nitrogen and branched chain fatty acid precursors) would ensure that these nutrients were not limiting (Mertens & Weimer, 1998).

Table 3.3 Constituents of the *in vitro* buffer solution.

Macro mineral	Reagents	1 L volume
	Distilled water	1000
	Na_2HPO_4 anhydrous (g)	5.7
	KH_2PO_4 anydrous (g)	6.2
	$MgSO_4.7H_2O$ (g)	0.59
	NaCl (g)	2.22
Micro mineral	Reagents	100 ml volume
	Distilled water (ml)	100
	$CaCl_2.2H_2O$ (g)	13.2
	$MnCl_2.4H_2O$ (g)	10
	$CoCl_2.6H_2O$ (g)	1
	$FeCl3.6H_2O$ (g)	8
Buffer solution	Reagents	1 L volume
	Distilled water (ml)	1000
	NH_4HCO_3 (g)	4
	$NaHCO_3$ (g)	35
Reducing solution	Reagents	100 ml volume
	Distilled water (ml)	100
	Cysteine Hydrochloric acid (g)	0.625
	KOH pellets (g)	10
	Na Sulphide non hydrate (g)	0.625

The medium was kept in a water bath at 39.0º C and mixed with the reducing solution while being flushed with CO_2. This was to enhance the mixture of the solution and to induce anaerobic conditions. The media was then sealed and left in the water bath at 39.0º C to reduce. The reduction of the buffer can be monitored by watching for a change in colour from a red or purple (oxidized) to a colourless solution (reduced) (Goering & Van Soest, 1970). The maintenance of temperature at 39.5º C as well as the reduced state of the buffer solution would respectively limit temperature and aerobic shock to rumen microbes when rumen fluid is mixed with the buffer solution (Mertens & Weimer, 1998). This would shorten the resulting lag phase experienced in terms of substrate degradation. As recommended by Tilley & Terry (1963), a ratio of 40:10 ml of reduced media to rumen liquor is adequate to maintain a pH ambiance within the usual limits for digestion to ensure that the final acid concentration does not exceed that found in the animal.

5. Collection and preparation of rumen fluid

The collection procedure of the rumen liquor (or rumen fluid) to be utilized in an *in vitro* system is of importance. Any stress (temperature change and O_2 presence) on anaerobic rumen microbes would directly affect the fermentation and thus the amount of GP or digestibility results. This is due to lower microbial concentrations to begin with in an *in vitro* system compared to the ruminal concentration of micro-organisms *in vivo* (Stern *et al*., 1997). Therefore any stress would negatively affect the microbial population with negatives consequences such as an increase of the lag period and decrease of the rate and extent of digestion. Considerations when collecting rumen liquor are:

♦ representative sampling of the rumen contents;
♦ collection of rumen solids to ensure the inclusion of fibrolytic microbes;
♦ maintaining anaerobic conditions of the rumen liquor;
♦ maintaining the temperature at 39º C;
♦ decreasing time that the rumen liquor is exposed to potential stresses such as O_2 presence, low temperature.

Rumen liquor was collected at 06h00, one hour after the morning feeding. Mauricio *et al*. (1999) reported that variation between rumen fluid harvested pre- and post feeding is negligible. In addition, the rumen liquor collected 2 to 4 hours post feeding can have an increased concentration of micro-organisms, with saccharolytic and amylolytic microbes being the most abundant. However, the increase of rumen microbes tends to be diluted in the rumen by high concentration of feed (Mauricio *et al*., 1999). This could result in an increased GP resulting from the rumen inoculum. Rumen content was squeezed through two layers of cheese cloth into pre-warmed flasks and a small amount of inoculum was added. The flasks were completely filled before being capped to keep the anaerobic milieu while they were transported to the laboratory. The rumen fluid with inoculum was blended in a pre-warmed industrial blender (Waring Commercial® Heavy Duty Blender, Waring® Corporation, New Hartford, CT, USA), at a low speed for 10 seconds. The rumen liquor required for the *in vitro* studies, which was run in parallel with an *in situ* incubation, was separately collected and kept in different flasks due to EFE treatments applied to feed before feeding. These were further blended and kept separately until their mixture to the reduced buffer solution. The reason for the blending procedure of the rumen liquor was to free bacteria that may be attached to solids (Goering & Van Soest, 1970). The rumen fluid was then filtered through two layers of cheese cloth into beakers and maintained at 39º C in the water bath while being flushed with carbon dioxide (CO_2) to sustain anaerobic condition. The strained inoculum was sealed and kept in an incubator at 39º C. A 20 mm magnetic stirring bar was placed in the beaker to maintain constant distribution of microbes in the liquor.

6. *In vitro* gas production system

In vitro methods such as Tilley & Terry (1963) and the nylon bag technique make use of gravimetric measurements and measure disappearance of feed substrate. Carbohydrates that are fermented in an anaerobic milieu by rumen microbes produce volatile fatty acids (VFA), methane (CH_4), CO_2 and small amounts of hydrogen (H_2) gas (McDonald *et al*., 2002). Thus, the measurement of *in vitro* GP can be used to evaluate ruminant feedstuffs. The *in vitro* GP system only measures the ability of certain fermentable nutrients of organic matter (OM) of feedstuffs to ferment into gas since ash, which can vary between substrates, do not contribute to gas or VFA production (Williams, 2000).

Glass vials of 116.0 – 120.0 ml of volume were used in the *in vitro* GP technique. Feed samples of 0.5 ± 0.01 g were weighed into bottles containing a magnetic stirrer each. These bottles were then flushed with CO_2 after adding 40 ml of reduced buffer solution to each bottle. The bottles were closed and placed in a water

bath at 39.5º C until the medium was reduced (clear), after which the bottles were re-opened and 10 ml of rumen fluid added while flushing with CO_2. The bottles were then closed tightly with rubber stoppers, crimp sealed and connected via needles to a pressure transducer system in the incubator at 39º C. Three bottles with only rumen liquor and reduced buffer solution were also included in each test as blanks for correction of gas produced. The bottles were placed on magnetic plates, which ensured that the magnetic stirrers constantly stirred the incubation medium and sample. The reason for stirring is to simulate the rumen mixing as it would occur *in vivo* to bring micro-organisms into contact with substrate. All bottles were zeroed in terms of gas produced by opening their valves which were attached to a 21 gauge needle inserted through the rubber stopper before the beginning of the incubation. Forty eight hours were used as period of incubation and gas pressure was recorded automatically using a pressure transducer system (Eagle technology Ltd.) based on the methods by Pell & Schofied (1993). Gas pressure was released at different intervals (i.e 3, 6, 9, 12, 24 and 48 hours) to prevent pressure build up in the bottles.

Gas measurements recorded at each interval were in terms of pressure (psi units). The psi pressure was later converted into volume as millilitres (ml) of gas produced using a calibration curve and the subsequent regression equation of pressure against volume for each bottle. This is important to correct irregularities in the head space volume between bottles (Williams, 2000). Since ash do not contribute to gas or VFA production and can vary between substrates, correction of gas produced as per DM basis to as per organic matter (OM) weight was also made. The calibration curve and the subsequent regression equation of pressure against volume were studied on a characteristic GP test. This was performed to attain a similar head space, where thirty-four vials were filled with rumen fluid, buffer solution and the magnetic stirring bar. The bottles were then sealed with a rubber stopper and a crimp cap. Thereafter a known amount of CO_2 gas was injected in duplicate vials before an overnight incubation at 39º C. The amounts of gas injected ranged from 0 ml with increments up to 70 ml. The room temperature was also measured.

The following day the amount of gas injected was then corrected for the expansion of the gas from room temperature to 39º C. The following equation was used for correction:

$a=b*[(39 + 273.15)/(c + 273.15)]$, where:
a: volume added at 39º C
b: volume added at room temperature (ml)
c: room temperature (25º C)

The volume of gas added to each bottle at 39º C was then divided by the head space or known gas volume of the bottle to give the volume fraction. The pressure of each bottle was then measured. Once completed, the net pressure for each bottle was estimated. This was done by subtracting the average pressure measured for two bottles where gas was not added from all the other pressures measured as correction for the gas produced from the added inoculum and buffer solution. The volume fraction of each bottle was then plotted against the net pressure measured within each bottle. The calibration curve and the regression equation, as described by Goosen (2005), showed a good correlation (R^2 = 0.9904) between the net pressure measured

and the volume fraction of the bottle. Thus, the regression equation of y = 0.0977x was used as standard regression equation to convert the pressure readings measured experimentally to a volume fraction. This calculated volume fraction would then be multiplied by the head space or known gas phase volume of each bottle to give the volume of gas produced in millilitres as follows:

Pressure (ml) at time t = 1000 x (0.0977 x Net pressure x head space) / OM with:
Net pressure (psi units) at time t = Psi produced from substrate bottle – Psi from blank bottle;
Head space of bottle (ml) = volume vial – 52.5;
OM (g) = (100 – Ash) / (100 x DM).

The constant of 52.5 ml represents an average volume of 10 ml of rumen fluid, 40 ml of reduced buffer solution and feed substrate. After the 48 hours of incubation, fermentation was terminated by placing the bottles on ice. Contents were transferred to tubes for centrifugation prior to drying at 60º C for further analysis.

7. *In vitro* digestibility procedure

The *in vitro* digestibility was evaluated using a modified incubator. The procedure used was performed as described by the manufacturers (ANKOM® Technology Corp., Fairport, NY, USA) but with slight amendments on the preparation of samples, reduced buffer solution and collection of rumen fluid. The modified incubator consists of a large incubator that can accommodate nine flasks or digestion jars of two litres each. Each flask of two litres contains a 4:1 ratio of 1130 ml of reduced buffer solution to 270 ml of rumen liquor, where a maximum of 28 bags are suspended. The reduced buffer solution was formed of 1076 ml of medium and 54 ml of reducing solution. The incubator was maintained at 39º C. It contains an inside fan to allow for even distribution of the heat around the flasks. All bags into the jar were agitated by constant slow turning as to stimulate rumen contractions.

Nylon bags (Dacron bags, Part R510, 50 x 55 mm bags, ANKOM® Technology Corp., Fairport, NY, USA) were used for forage samples while the multi layer polyethylene polyester bags (ANKOM® F57 filter bag, ANKOM® Technology Corp., Fairport, NY, USA) were used for concentrate samples. The porosity of the F57 filter bag is 30 µm (ANKOM Technology Corporation, 1997), therefore small particles of less than 30 µm diameter can escape from the filter bag during digestion. The F57 bags were washed in acetone for three to five minutes to remove the barrier layer that limits the microbial penetration into the filter bag. Thereafter they were allowed to air dry. F57 bags and nylon bags were marked and placed in the drying oven at 100º C over night. When dried, they were placed in the desiccator before being weighted. Once completed, 0.5 ± 0.01 g of forage samples (lucerne hay, wheat straw or wheat straw treated with urea) or 0.25 ± 0.01 g of the concentrate diet were respectively filled into nylon bags and F57 bags and heat sealed using an impulse heat sealer (ANKOM® 1915/1920 Heat sealer; ANKOM® Technology Corp., Fairport, NY, USA). A quantity of 24 bags filled with substrates and 3 blank bags (containing no substrate) were accommodated into a jar,

ensuring that they were well distributed between both sides of the digestion jar divider. The reason for inserting blank bags as corrector was to account for weight changes due to microbial contamination happening during the incubation.

A pre-warmed (39º C) and reduced buffer solution (1130 ml) was poured into each jar, while flushing with CO_2. Each jar contained 27 filter bags which were pre treated 12 hours prior to incubation with enzyme dilution (28 ml). The jar was then sealed and placed in the water bath at 39º C to equilibrate the milieu. Rumen liquor (270 ml) was then added to each flask. The digestion jars were purged with CO_2 gas before being sealed and placed into the incubator in slow turning motion for digestion. Flushing CO_2 into the jar was to ensure anaerobic conditions. At defined periods of time as described in the following chapters, three bags were removed per jar. The jar with its remaining contents were flushed with CO_2 and returned to the incubator. The retrieved bags were gently washed under running cold water, before being frozen at -4º C until analyzed. When the trial was done, bags were defrosted at room temperature and washed mechanically until the running water was clear. Once spun to remove excess washing water, bags were placed in the drying oven at 60º C for three days. On completion of the drying period, bags were removed from the oven, placed in the desiccator for 30 minutes and weighed for DM estimation. Following DM determination from three bags retrieved at time 3, 6, 9, 12, 24 and 48 hours, one bag was later allocated for NDF determination while the remaining two were pooled together for further analysis (CP and purine derivates).

8. Chemical analysis of samples

Chemical analyses of feedstuffs were performed on the 2 mm milled and sieved samples and their residues after digestion. All results are expressed on a 100% dry matter (DM) basis. The DM of the original samples was obtained after drying at 105º C overnight (AOAC, 1995; Method 930.15). Organic matter (OM) was determined after ashing at 500º C in a muffle furnace for 6 hours (AOAC, 1995; Method 942.05). After digestion, sample residues were dried at 60º C for three days before DM determination. Neutral-detergent fibre (NDF) and acid-detergent fibre (ADF) were estimated by using ANKOM$^{200/220}$ Fibre analyzer (ANKOM® Technology Corp., Fairport, NY, USA). The NDF component was determined on 0.5 g of each original sample into separate F57 ANKOM or nylon fibre analysis bags and their relative residues after incubation as described by the manufacturers. The bags were heat sealed and NDF was determined using the method of Van Soest *et al*. (1991). The sodium sulphite anhydrous (Na_2SO_3) was added to the NDF solution during extraction and heat-stable α-amylase was added during rinsing with warm water. The ADF was also determined using the method of Van Soest *et al*. (1991)

Total nitrogen content was determined using the Nitrogen gas analyzer (FP-528 Protein/Nitrogen determinator, St Joseph, Leco© Corporation, USA). About 0.1 g of sample was weighed into a small piece of aluminium foil. The samples were then ignited in a furnace at 900º C using the Dumas procedure (AOAC, 1990; Method 968.06). Crude protein (CP) was obtained by multiplying N content by 6.25 (AOAC, 1995; method 990.03). The microbial protein synthesis (MPS) on feed residues after digestion was measured as

purine derivates (µg RNA equivalent/g DM) according to Zinn & Owens (1986). This method consists of an extraction of purine bases by $HClO_4$ followed by their precipitation with $AgNO_3$. In short, 0.25 ± 0.01 g digested residue was placed into a 25 mm width screw-cap Pyrex tube and 2.5 ml $HClO_4$ (70% A.R.) was added. The mixture was covered and incubated in a water bath at 90-95º C for one hour. After cooling, tubes were opened and pellets were broken using a glass rod for a complete extraction. Quantities of 17.5 ml of 0.0285 M $NH_4H_2PO_4$ were added and tubes were returned to the water bath (90-95º C) for 30 minutes. After cooling, the contents were filtered twice through Whatman No.4 filter paper. One ml filtrate was transferred to a 15 ml tube and 0.5 ml $AgNO_3$ (0.4 M) and 8.5 ml $NH_4H_2PO_4$ (0.2 M) were added. Tubes were screw capped and allowed to stand overnight at 4º C. The contents were centrifuged at 4000 rpm for 15 minutes and the supernatant fraction was discarded with care as to not disturb the pellet. The pellet was broken with a glass rod and washed with 5 ml of the pH 2 distilled water (with H_2SO_4) followed by centrifugation at 4000 rpm for 15 minutes (at 4º C). After the supernatant was discarded, the pellet was broken with a glass rod, suspended in 10 ml 0.5 N HCl, vortex-mixed thoroughly and transferred into a 25 mm width screw cap tube. These tubes were screwed capped and placed in the water bath (90-95º C) for 30 minutes. After cooling, the content was centrifuged at 4000 rpm for 15 minutes (at 4º C) and the absorbance of the supernatant fraction was recorded at 260 ŋm against 0.5 N HCl. A standard of 0.05 g yeast RNA (93% CP), treated as described above but diluted according to AOAC (1995) just before the incubation in the water bath using 0.5 N HCl as diluent, was used in this method.

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CHAPTER 4

Screening of exogenous fibrolytic enzymes

Abstract

The use of exogenous fibrolytic enzymes (EFE) in ruminant systems has shown promises to increase forage utilization, improve production efficiency and reduce nutrient excretion. However, the effectiveness of EFE products is highly variable. Part of this variability may be due to the specificity of the enzyme products for different feed types. An assessment of EFE (Abo 374, EFE 2 and EFE 3) and the microbial yeast preparation (3.3×10^9 coliform units, CFU/g) was conducted for the potential to improve fibre digestion in the rumen using an *in vitro* gas production (GP) technique. The feed substrates used in the screening were lucerne hay, wheat straw, wheat straw treated with urea and a commercial concentrate diet. The 24 hours cumulative GP was used as a screening step to identify the promising EFE. Results showed that EFE 3 had low response compared to other treatments when tested on different substrates. Thus, two EFE products (Abo 374 and EFE 2) and the microbial yeast preparation were then identified and selected for their potential to improve *in vitro* fermentation. These treatments may be compatible to the chemical composition of the targeted substrates, due to their substrate specificity. As a rough tool of selection, the GP system had likely identified Abo 374, EFE 2 and the microbial yeast preparation in terms of characterization of biotechnological products and feed specificity for further investigations.

Key words: exogenous fibrolytic enzymes (EFE), dry matter (DM), gas production (GP), neutral-detergent fibre (NDF).

Introduction

The treatment of forages with biotechnological products such as exogenous fibrolytic enzymes (EFE) has shown promise at hydrolyzing plant cell walls (Beauchemin *et al*., 2003). Studies have reported increases in DM digestion *in situ* and *in vivo* (Feng *et al*., 1996; Yang *et al*., 1999; Cruywagen & Goosen, 2004; Cruywagen & Van Zyl, 2008) and in voluntary intake (Feng *et al*., 1996; Pinos-Rodríguez *et al*., 2002) when EFE were added to ruminant diets. Although positive effects with EFE had been observed in some studies, others reported negative or no effects (ZoBell *et al*., 2000; Vicini *et al*., 2003). This variability in effectiveness of EFE was attributable partly to the specificity of the enzyme products for different feed types (Beauchemin *et al*., 1995). Most commercial EFE are complex products produced for non-feed applications, which include food, pulp and paper, textile, fuel and chemical industries (Bhat, 2000). In ruminant systems, EFE are expected to act through direct hydrolysis, enhancement of microbial attachment and synergy with the endogenous enzyme activities of the rumen microbes (McAllister *et al*., 2001). Thus, the key activities needed to improve forage fibre degradation likely differ from those needed for other applications (Wallace *et al*., 2001; Colombatto *et al*., 2003). In addition, the identification of the key activities needed for EFE to be consistently effective in ruminants is still a challenge. This is because the mechanisms whereby EFE enhance microbial digestion of feed are not well understood (Beauchemin *et al*., 2004). In an attempt to establish the enzyme-feed substrate specificity, an assay was performed on a range of feed substrates using the *in vitro* GP system to screen the EFE (Abo 374, EFE 2 and EFE 3) and microbial yeast for the potential to increase fermentation parameters.

Materials and methods

Three potential EFE (Abo 374, EFE 2 and EFE 3) and a microbial yeast preparation (M-yeast) were evaluated on four different substrates using an *in vitro* fermentation system. Abo 374 is a South African fungal EFE cultivated on wheat straw and developed at the Department of Microbiology (Stellenbosch University). Both EFE 2 (Cattle-Ase™, Loveland Industries Inc., Greeley, CO, USA) and EFE 3 (Pentopan® Mono BG, Novozymes, Denmark) are commercial products. The microbial yeast preparation (Levucell© SC, Lallemand Animal nutrition, USA) is a commercial direct-fed microbial product containing *Saccharomyces cerevisiae* at a ratio of 3.3 x 10^9 coliform units (CFU)/g. The feed substrates used in the screening were lucerne hay, wheat straw, wheat straw treated with urea and a commercial concentrate diet (306.5, 709.43, 760.6 and 242.38 g NDF / kg DM, respectively). As described in Chapter 2, the GP technique based on Tilley & Terry (1963) was conducted with rumen liquor collected at 06h00 on fistulated Döhne-Merino sheep according to the protocol of the animal care and use committee of Stellenbosch University (SU ACUC, Ethic clearance number: 2006B03005). These animals were maintained on a standard forage-based diet (basal diet A), fed *ad libitum*. Treatments were applied 12 hours prior to incubation to allow an enzyme-substrate interaction time (Beauchemin *et al*., 2003) at a ratio of 1ml of a particular treatment dilution to 0.5 g substrate as reported in Chapter three. The cumulative GP (based on the Reading pressure technique) at 24 hours was used as a screening step to identify superior EFE products. As the net effect of EFE is known to stimulate the initial phases of substrate degradation (Nsereko *et al*., 2000; Colombatto *et al*., 2003), it was thus deemed sufficient for screening purposes to limit the incubation to 24 hours. Simple statistical analyses were performed to determine the average values and standard error values, but no tests were done to estimate whether observed differences were significant, due to insufficient replications. The selection was therefore based on the ability of the treatment to enhance the 24 hours cumulative GP.

Results and discussion

The results of the cumulative GP of the EFE screening at 24 hours are presented in Figure 4.1. The cumulative GP with EFE 3 was the lowest on lucerne hay, wheat straw treated with urea and concentrate diet. Eun & Beauchemin (2007) assessed the potential of different endoglucanases and xylanases exhibiting different biochemical properties using the cumulative GP at 18 hours. Their results revealed that EFE on lucerne hay can improve the GP. Eun *et al*. (2007) also found that EFE substantially improved the cumulative GP and fibre degradation of lucerne hay and corn silage at 24 hours. In another study, Kung *et al*. (2002) found that the *in vitro* GP from forages treated with EFE was significantly higher than from untreated forage. In agreement with these findings, the present screening revealed that the cumulative GP at 24 hours was observably improved on lucerne hay, wheat straw and concentrate diet following EFE addition.

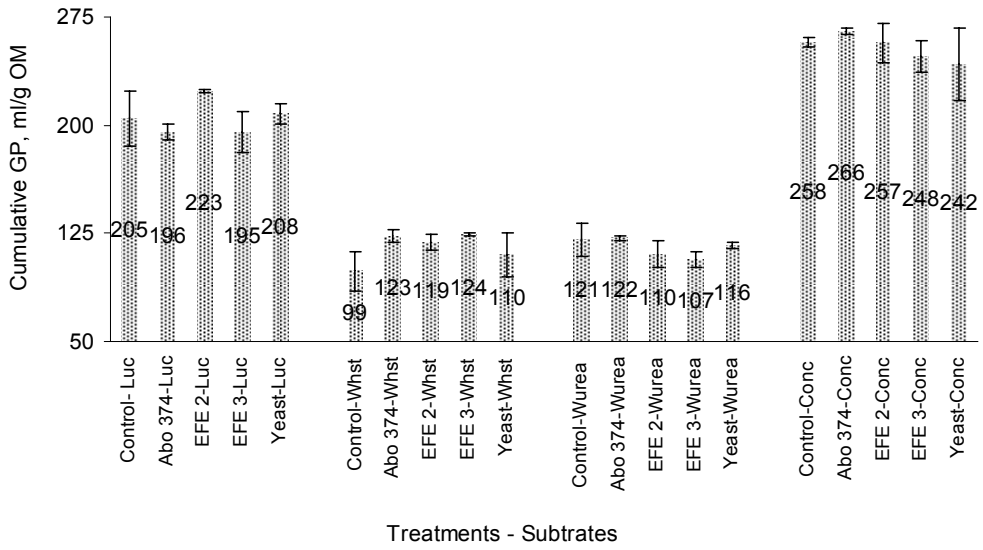

Figure 4.1 Cumulative GP at 24 hours (ml/g OM) of different substrates (Luc: lucerne hay, Whst: wheat straw, Wurea: wheat straw treated with urea and Conc: concentrate diet). Substrates were incubated with buffered rumen fluid and EFE (Abo 374, EFE 2 or EFE 3) or microbial yeast preparation for 24 hours. Error bars indicate the standard error of means (s.e.m).

Goosen (2005) found that adding Abo 374 to wheat straw can improve the net cumulative GP and DM disappearance by > 10% (at 18 hours). Similarly, Abo 374 on wheat straw improved the cumulative GP by 24.24% at 24 hours in this study. This suggests that the Reading pressure technique identified slight changes in fermentation of different substrates due to enzyme-feed substrate affinity. Thus, two EFE (Abo 374, EFE 2) products and the microbial yeast (3.3 x 10^9 CFU/g) were then identified for their potential to improve GP. The key enzymatic activities of EFE, which is a major factor at improving hydrolysis of plant cell walls, may differ among feedstuff substrates (Wallace *et al*., 2001; Colombatto *et al*., 2003). With regard to such hypothesis, Wallace *et al.* (2001) found that EFE with high endoglucanase activity increased the rate of GP from corn silage compared to no enzyme, but EFE with a high xylanase activity did not. In another case, a high correlation between added EFE with endoglucanase activity and OM degradation enhancement was found for lucerne hay (Eun & Beauchemin, 2007). It could be speculated that the array of activities of Abo 374, EFE 2 and the microbial yeast preparation were compatible to the chemical composition of the targeted substrates, probably due to their specificity for substrate.

Conclusion

The use of biotechnology such as EFE to enhance quality and digestibility of fibrous forage is on the verge of delivering practical benefits to ruminant production systems. However the enzyme-feed specificity presents a part of the major dilemma with ruminant EFE products as ruminant diets are composed of complex plant cell wall materials from several types of forages and concentrates. This impairs the understanding of what

mechanism of action is behind the relationship between enzymatic activities and improvement in forage utilization in ruminant systems. The EFE 3 had low GP response at 24 hours compared to other treatments relative to control when tested on different substrates. Despite the difficulty to accurately predict the performance of a given enzyme additive based only on cumulative GP, the system can be useful as a preliminary *in vitro* indicator. As a rough tool of selection, it had the potential to identify Abo 374, EFE 2 and the microbial yeast preparation in terms of characterization of biotechnological products and feed specificity for further evaluation studies.

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CHAPTER 5

Effect of exogenous fibrolytic enzymes on crude protein (N) and fibre digestion using two *in vitro* evaluation techniques

Abstract

Ruminants make up a significant proportion of the domesticated animal species worldwide. Amongst the farmed livestock, ruminants are the best adapted to utilization of plant cell walls. The lignocellulose components, which represent the most renewable carbon source on earth, are both economical as feedstuffs and necessary for normal healthy rumen function. In addition, following ruminal fermentation, fibre yields volatile fatty acids (VFA) and contributes towards the synthesis of microbial protein (MPS). The VFA are absorbed through the rumen wall and constitute the major metabolic fuel for the host animal. On the other hand, the MPS represent a significant source of protein and amino acids when digested in the small intestine. Improvements in the ability of ruminal micro-organisms to degrade plant cell walls are generally highly desirable and usually lead to improved animal performance. Therefore, this study was undertaken to improve the digestion of plant cell walls using exogenous fibrolytic enzymes (EFE). Hence, two EFE (Abo 374 and EFE 2) products were assessed *in vitro* for their impact on MPS and disappearances of DM, CP and NDF. Abo 374, EFE 2 and the microbial yeast preparation were tested on four different substrates (lucerne hay, wheat straw, wheat straw treated with urea and a commercial concentrate diet), using the *in vitro* gas production (GP) system and the ANKOM digestion technique. The *in vitro* GP and ANKOM digestion were simultaneously conducted using the reduced buffer solution and rumen fluid prepared and collected at the same time. The rumen liquor required for these incubations was obtained from cannulated sheep maintained on a standard forage-based diet.

The EFE significantly increased the cumulative GP ($P < 0.05$), but no correlation between the GP and MPS ($P < 0.05$; $R^2 < 0.30$), estimated by purine derivates, was observed with all substrates tested. Abo 374 significantly augmented MPS on the concentrate diet when evaluated with the residues of GP ($P < 0.0001$). Abo 374 significantly increased the *in vitro* NDF disappearance of lucerne hay ($P < 0.0001$). The EFE and the microbial yeast preparation did not improve *in vitro* NDF disappearance of wheat straw, wheat straw treated with urea and concentrate diet, but significantly increased *in vitro* DM disappearance of all four substrates at 48 hours ($P < 0.05$) with Abo 374 being the best treatment. Abo 374 and the yeast preparation had a significant effect on CP disappearance of wheat straw and concentrate diet ($P < 0.05$). The MPS of all the substrates was significantly increased during the first half-period of incubation with EFE treatments using the *in vitro* filter bag procedure ($P < 0.05$). However, the observed MPS responses were likely variable as a result of the poor recovery of purine derivates with the Zinn & Owen (1986) analysis procedures and possible microbial lysis with long periods of incubation. Results showed that EFE can affect the degradability of CP and the output of MPS in addition to the enhanced DM and NDF disappearances and the improved GP profiles. Direct hydrolysis of fibrous fractions due to EFE addition during the pre-treatment period may have initiated erosive alterations of the network of plant cell walls, thereby making it more susceptible to microbial degradation. As indicated by the higher MPS observed during digestion, it could be speculated that the improvement in GP and disappearance of DM were obtained throughout a combined effect of direct enzyme hydrolysis and synergetic effect between exogenous (applied) and endogenous (rumen) fibrolytic enzymes.

Key words: crude protein (CP), exogenous fibrolytic enzymes (EFE), dry matter (DM), gas production (GP), neutral-detergent fibre (NDF), microbial protein synthesis (MPS).

Introduction

Ruminant agriculture is dependant on forages. The economic implications of roughage-based diets in ruminant nutrition are undisputable. However, only 10 to 35% of energy intake is retained as net energy (Varga & Kolver, 1997) under ideal rumen conditions because cell wall digestion is not totally efficient (Krause *et al*., 2003). With plant cell walls contributing up to 70% of forage dry matter (Van Soest, 1994), the attempt to improve fibre digestion in the rumen is still an active research area. Many methodologies have been developed to improve forage quality in ruminant systems. These strategies have consisted of the plant breeding and management for improved digestibility (Casler & Vogel, 1999) and the increase of utilization by physical, chemical and/or biotechnological actions (McDonald *et al*, 2002). Despite enhancements achieved through these strategies, forage digestibility continues to limit the intake of digestible energy in ruminants because not even 50% of this fraction is readily digested and utilized (Hatfield *et al.*, 1999).

Recent advances in fermentation technology and biotechnology have permitted the incorporation of EFE as feed additives to improve fibre digestion. The ability of EFE at improving fibre digestibility, which can thus enhance the amount of available digestible energy, has been studied using *in vitro*, *in situ* or *in vivo* systems. Many positive responses on animal production traits have been reported with EFE as a result of increased microbial activity in the rumen (Lewis *et al*., 1999; Rode *et al*., 1999; Yang *et al*., 1999; Beauchemin *et al*. 2003; Cruywagen & Goosen, 2004; Balci *et al*., 2007; Cruywagen & Van Zyl, 2008; Bala *et al*., 2009). However, EFE addition in some other studies had negative effects or none at all (Bowman *et al*., 2003; Vicini *et al*, 2003; Baloyi, 2008). These variations in EFE responses might be attributable to differences in enzyme type, preparation, activity, application rate (Bowman *et al*., 2003; Beauchemin *et al*., 2003), mode of application or the portion of the diet to which the enzyme was added (Feng *et al*., 1996; Lewis *et al*., 1996; ZoBell *et al*., 2000) and experimental conditions (Beauchemin *et al*., 2003).

In vivo experimentations involving animals provide the most accurate methods to estimate the effects of EFE in the rumen, but they may not be useful for comparison purpose due to a number of restrictions and difficulties. These include the time needed to perform animal trials, costs related to feeding and care of animals, number of animals needed to reach significant results and restrictions regarding the amount of treatments such trials can accommodate at one time (Mohamed & Chauldry, 2008). The use of biological laboratory methods that simulate the ruminal digestion therefore represents a cost and time-effective alternative to *in vivo* trials. However, these methods (*in vitro* gas production system and nylon bag technique) are imperfect by nature for multiple reasons, to measure the rumen activity. These involve the straining of rumen liquor (or rumen fluid) before being used, diluting and heavy buffering of rumen liquor, pooling together rumen fluid from several animals before use (Wallace *et al*., 2001) and so forth. Nevertheless, the *in vitro* techniques are convenient to use as first approximations and they are particularly useful for comparative purposes (Wallace *et al*., 2001). The *in vitro* true digestibility technique is both repeatable as well as being closely related to the *in situ* digestibility (Spanghero *et al*., 2003). The *in vitro* gas production (GP) can be utilized to determine the fermentation characteristics of large numbers of samples at accurately maintainable

experimental conditions (Getachew *et al*., 1998). These techniques can be useful as preliminary *in vitro* indicators to identify and select promising EFE before further testing *in vivo*.

In an attempt to improve the digestibility of ruminant feeds, two EFE (Abo 374 and EFE 2) and one yeast preparation of 3.3 x 10^9 coliform units (CFU)/g were evaluated for their potential to affect rumen protein degradation and fibre digestion of four different substrates and subsequently to improve microbial protein synthesis (MPS). Specific objectives of this trial were to:

1) evaluate EFE and the microbial yeast preparation for their impacts on MPS and digestibility parameters in the rumen using the GP profiles and *in vitro* nylon bag technique;
2) and to determine the relationship between MPS and the cumulative GP at 48 hours of incubation of lucerne hay, wheat straw, wheat straw treated with urea and a commercial concentrate diet substrates in buffered rumen fluid.

Materials and methods

Based on the protocols of the animal care and use committee of Stellenbosch University (SU ACUC, Ethic clearance number: 2006B03005), four cannulated Döhne-Merino sheep were used in this investigation to evaluate the effects of EFE on a range of different substrates using *in vitro* techniques. The feed substrates used were lucerne hay, wheat straw, wheat straw treated with urea and a commercial concentrate diet (306.5, 709.43, 760.6 and 242.38 g NDF /kg DM, respectively. Table 3.1, Chapter three). The animals were randomly assigned to two groups. One group was used in trials for rumen collection while the other group was on Kikuyu pasture to avoid long animal containment in pens. Sheep in holding pens received a basal diet A fed *ad libitum* composed of a standard forage-based diet [lucerne hay and wheat straw at 1:1 ratio and 0.5% urea in premix] supplemented with 300 g/day of concentrate [880 g/kg of DM, 120 g/kg of CP, 200 g/kg of NDF, 10 g/kg Ca and 8 g/kg of P]. Water was offered *ad libitum*. The concentrate diet was given in the morning. An adaptation of ten days to the basal diet was allowed prior to the collection of rumen liquor. According to methodology described in Chapter three, the *in vitro* filter bag technique (ANKOM Technology Corp., Fairport, NY, USA) and the *in vitro* GP system were simultaneously conducted with the reduced buffer solution prepared at the same time and the rumen liquor collected at 06h00.

Treatments consisted of Abo 374, EFE 2, yeast preparation (M-yeast) and control (distilled water). Abo 374 is a South African fungal EFE cultivated on wheat straw and developed at the Department of Microbiology (Stellenbosch University). EFE 2 (Cattle-Ase™, Loveland Industries Inc., Greeley, CO, USA) is a commercial EFE product. The yeast preparation (Levucell© SC, Lallemand Animal nutrition, USA) is a commercial direct-fed microbial product containing *Saccharomyces cerevisiae* at a ratio of 3.3 x 10^9 CFU/g. Treatments were applied 12 hours prior to incubation to allow an enzyme-substrate interaction time (Beauchemin *et al*., 2003) at a ratio of 1 ml of a particular treatment dilution to 0.5 g substrate as reported in Chapter three. After 48 hours of *in vitro* digestion, the disappearance (DM, NDF and CP) and MPS (as purine derivates) were

estimated on residues of incubations according to chemical analyses described in Chapter three. Data generated from the digestibility studies was therefore subjected to the two-way repeated measures of analysis of variance (ANOVA) using SAS enterprise guide 4 (2006, SAS Institute Inc.). The model includes the treatment effect, time effect of observation and interaction effect of treatment and time as fixed effects, whereas animal influence within treatments was specified as a random effect. The measured variables obtained at each time (i.e 0, 3, 6, 9, 12, 24 and 48) during the 48 hours incubations were considered as repeated observations of a particular block. The model was defined as follow:

$Y = \mu + \alpha_i + ß_j + (\alpha ß)_{ij} + \delta_{(ij)k} + \varepsilon_{ijk}$ where

μ = overall mean;

α_i = i^{th} level of treatment factor (main effect);

$ß_j$ = j^{th} level of time factor (main effect);

$(\alpha ß)_{ij}$ = interaction between level i of treatment and j of time (interaction effect);

$\delta_{(ij)k}$ = effect of the k^{th} block effect in the ith treatment (variable effect);

ε_{ijk} = I,j,k^{th} error term.

The assumptions were described as $\Sigma i\ \alpha_i$, $\Sigma j\ ß_j$ and $\Sigma i\ (\alpha ß)_{ij} = \Sigma i\ (\alpha ß)_{ij}$ equal to zero with $\delta_{(ij)k} \sim N(0, \sigma_e^2)$ varying independently of ε_{ijk}. Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test. Significance was declared at $P < 0.05$. The relationship between the cumulative GP and MPS at 48 hours of incubation was studied using the correlation analysis (Pearson test) of SAS enterprise guide 4. The fitting of the non-linear model $Y= a + b (1- \exp^{-ct})$ as described by Ørskov & McDonald (1979) on the digestion profiles of lucerne hay taken as the reference did not provide similar ruminal degradation a, b or c parameters as reported in literature (Bangani, 2002) because incubations were terminated after 48 hours. Inaccuracy of parameters may be due to the short period digestion profiles used in this study as this model was conceived with long period digestion profiles (96 hours) reaching the stationary phase of rumen degradation. To estimate the rate of degradation, data were fitted to non-linear model using the quadratic fit $Y = a + bx + cx^2$, with *x* being the time factor. This was done in consultation with the Department of Statistics (Stellenbosch University). Estimates a, b, c and R^2 value for quadratic function were found using the Prog. GLM of SAS enterprise guide 4 and the instantaneous rate was found from the derivate function of the quadratic function $f'(x) = b + 2cx$.

Results and discussion

The effects of different feed substrates on *in vitro* cumulative GP at 48 hours (regardless of treatments of EFE and microbial yeast) revealed significant differences ($P < 0.0001$) (Figure 5.1). The means of cumulative GP ranged from 316.42 ml/g OM for the concentrate diet to 134.46 ml/g OM for wheat straw treated with urea ($P < 0.0001$). The amount of gas produced by feedstuffs varied with substrate type. Dijsktra *et al*. (2005) reported that the amount of gas produced by a feed substrate depends on its energy concentration and chemical composition. Consistent with this hypothesis, Menke & Steingass (1987), as cited by

Krishnamoorthy *et al*. (1991), observed that the amount of gas produced per unit of carbohydrate fermented differs according to different biochemical pathways of structural and non-structural carbohydrate fermentation. In this study, wheat straw with its high fibre content (709 g NDF /kg DM) was less fermentable than the commercial concentrate diet (242 g NDF /kg DM) followed by lucerne hay (306 g NDF /kg DM). Treating wheat straw with urea decreased the cumulative GP by 21.38% compared to wheat straw. This was possibly due to the toxic effects of ammonia on rumen bacteria *in vitro* because treating wheat straw with 2% urea supplied 20g urea /kg of feed. Satter & Slyter (1974) reported that the concentration at which ammonia nitrogen (NH_3-N) became limiting for rumen bacteria maintained in an *in vitro* steady state condition was 5 mg/100 ml of rumen fluid or less. This stipulated that no more than 2.24% nitrogen (14% crude protein equivalent, CPE) was required to reach this concentration of ammonia *in vitro* with diets containing less than 30% fibre. In addition, less digestible diets were found to require less dietary nitrogen to maintain the required ammonia for maximum ruminal MPS (Slyter *et al.,* 1979). Therefore, high N content coupled with poor readily available energy in wheat straw treated with urea to enhance microbial protein synthesis could have likely resulted in reduced MPS.

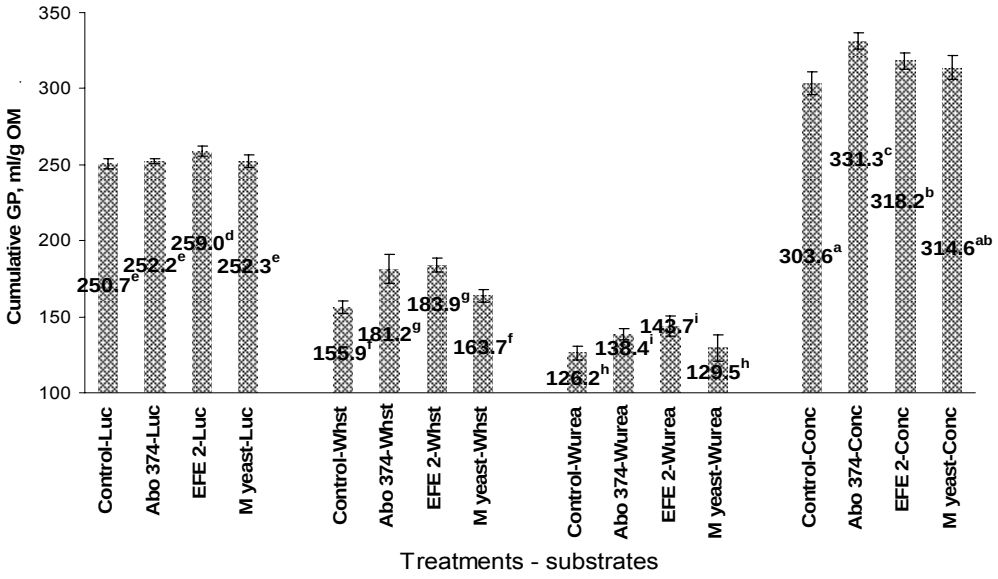

Figure 5.1 Cumulative GP (ml/g OM) of different substrates at 48 hours (Luc: lucerne hay, Whst: wheat straw, Wurea: wheat straw treated with urea and Conc: concentrate diet). Substrates were incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation for 48 hours. Error bars indicate the standard error of means (s.e.m).

The effects of EFE and the microbial yeast preparation (3.3 x 10^9 CFU/g) on *in vitro* cumulative GP and DMD, CP degradation and MPS (measured as purine derivates) on GP residues of different substrates (lucerne hay, wheat straw, wheat straw treated with urea and a concentrate diet) are presented in Tables 5.1-4 and Figures 5.2-5. Results showed that the GP profiles of all four different substrates were significantly influenced by treatments ($P < 0.05$) and were changed in quadratic trend with advancing time ($P < 0.0001$). In general, treatment effects were not effective in the early phase of digestion. Except for wheat straw ($P = 0.0137$), the

interactive effects of treatment and time were not significant on the GP profiles of lucerne hay, wheat straw treated with urea and concentrate diet.

Table 5.1 Cumulative GP profiles, DMD, CP degradation and MPS (measured as purine derivates) on GP residues of lucerne hay.

Lucerne	Cumulative gas production, ml/g OM											
Time, hours	Control			Abo 374			EFE 2			Yeast preparation		
1.5	21.34	±	0.66	21.58	±	1.11	20.83	±	0.97	22.81	±	0.55
3	45.34	±	0.62	45.40	±	1.11	46.32	±	0.93	47.83	±	1.63
6	106.24	±	1.43	105.97	±	1.87	109.32	±	2.85	107.74	±	2.74
9	151.34	±	2.20	151.36	±	2.21	156.51	±	3.97	153.19	±	3.71
12	178.30	±	1.38	176.31	±	2.11	181.68	±	3.33	178.24	±	2.93
24	225.99^{a}	±	2.30	226.44a^{b}	±	1.52	232.04^{b}	±	2.41	227.01^{ab}	±	2.46
36	243.90^{a}	±	3.22	244.81^{a}	±	1.83	251.73^{b}	±	3.62	244.72^{a}	±	3.91
48	250.70^{a}	±	3.40	252.15^{a}	±	1.83	258.98^{b}	±	3.55	252.28^{a}	±	3.79
ANOVA GP	Treatment		Time	Treatment X Time								
P values	0.0009		<0.0001	0.8667								
	Control			Abo 374			EFE 2			Yeast preparation		P values
MPS, µg RNA/g	143.8	±	8.9	165.6	±	7.5	144.5	±	8.5	151.8	±	8	0.2308
DMD, %	55.7	±	0.6	56.7	±	0.5	55.9	±	1.8	56.8	±	1.5	0.9
CP degradat, %	51.4	±	1.5	49.9	±	1.6	50.1	±	0.9	52.5	±	1.3	0.5

Means (± standard error mean, s.e.m) within rows with different superscript letters differ (P ≤ 0.05).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test.

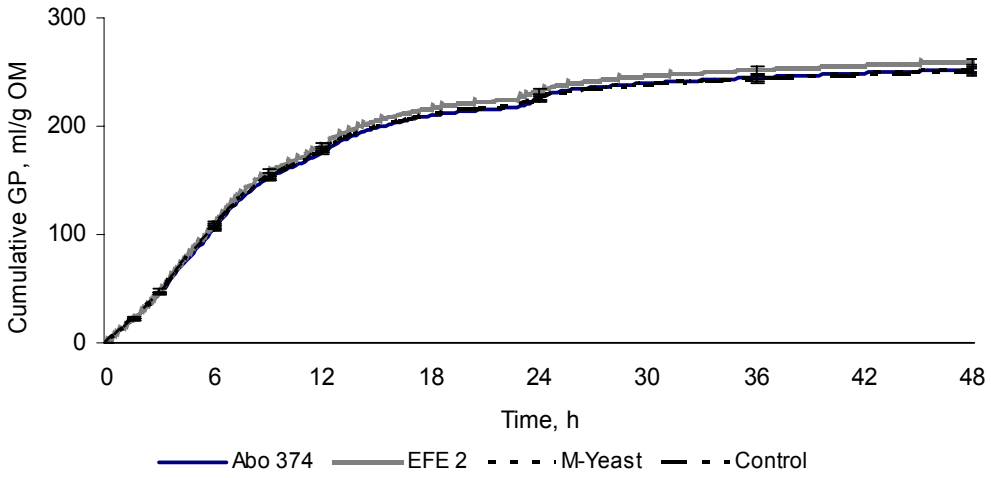

Figure 5.2 Cumulative GP profiles* of lucerne hay incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 20.65 + 13.64 time – 0.19 time^2, R^2 = 0.95; GP (Abo 374) = 20.55 + 13.61 time – 0.19 time^2, R^2 = 0.95; GP (EFE 2) = 20.69 + 14.04 time – 0.19 time^2, R^2 = 0.95; GP (Yeast prep.) = 22.69 + 13.55 time – 0.19 time^2, R^2 = 0.95.

Table 5.2 Cumulative GP profiles, DMD and MPS (measured as purine derivates) on GP residues of wheat straw.

Wheat straw	Cumulative GP, ml/g OM												
Time, hours	Control			Abo 374			EFE 2			Yeast preparation			
1.5	4.64	±	1.54	7.99	±	1.45	8.67	±	1.10	5.60	±	1.13	
3	8.90	±	2.35	13.90	±	2.27	13.90	±	1.96	11.04	±	1.79	
6	18.68	±	3.34	27.02	±	3.09	27.29	±	2.49	23.76	±	2.27	
9	28.50^{a}	±	4.91	42.26^{b}	±	5.09	42.37^{b}	±	4.26	36.27^{ab}	±	3.20	
12	42.52^{a}	±	6.04	58.81^{ab}	±	5.69	61.38^{b}	±	4.99	51.26^{a}	±	3.82	
24	106.23^{a}	±	4.09	122.05^{b}	±	6.71	129.51^{b}	±	3.26	111.53^{a}	±	3.00	
36	138.31^{a}	±	3.87	160.01^{b}	±	8.72	164.96^{b}	±	3.87	144.07^{a}	±	3.51	
48	155.91^{a}	±	4.14	181.25^{b}	±	9.60	183.91^{b}	±	4.61	163.73^{a}	±	4.16	
ANOVA GP	Treatment		Time	Treatment X Time									
P values	<0.0001		<0.0001	0.0137									
	Control			Abo 374			EFE 2			Yeast preparation			P values
MPS, µg RNA/g	88.36	±	4.25	96.69	±	4.32	94.9	±	5.65	89.58	±	3.99	0.5184
DMD, %	24.95	±	3.3	29.01	±	1.61	28.23	±	3.68	27.25	±	4.32	0.8442
CP degradat*, %		-			-			-			-		

*CP degradation of wheat straw was biased due the microbial contamination in the gas production system
Means (± standard error mean, s.e.m) within rows with different superscript letters differ (P ≤ 0.05).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test

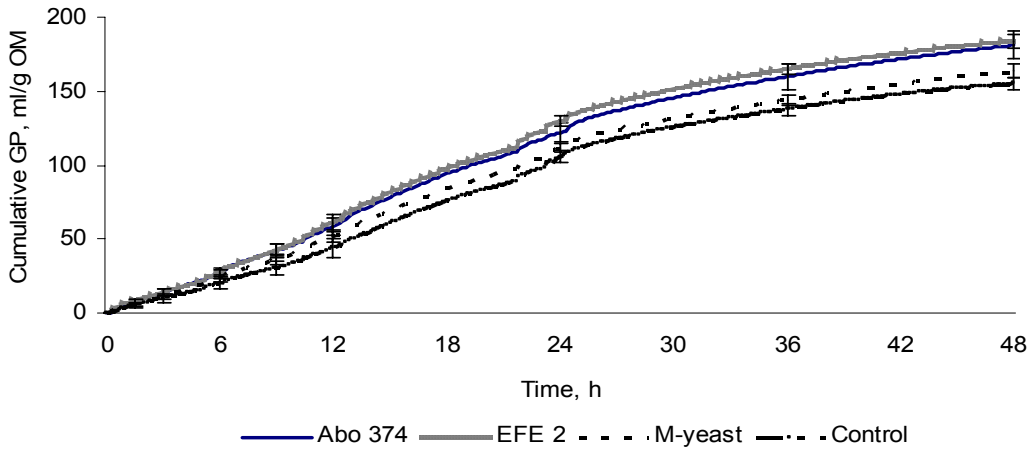

Figure 5.3 Cumulative GP profiles* of wheat straw incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = - 9.2 + 5.52 time – 0.042 time^2, R^2 = 0.96; GP (Abo 374) = - 6.6 + 6.4 time – 0.051 time^2, R^2 = 0.94; GP (EFE 2) = - 8.27 + 6.9 time – 0.06 time^2, R^2 = 0.97; GP (Yeast prep.) = - 7.94 + 5.90 time – 0.048 time^2, R^2 = 0.98.

The GP of lucerne hay was significantly improved with EFE 2 enzyme from 24 to 48 hours, with improvements of 3.3% at 48 hours (Table 5.1 and Figure 5.2). EFE increased the GP of wheat straw, wheat straw treated with urea and concentrate diet (P < 0.05). Abo 374 and EFE 2 improved the GP of wheat straw from 9 hours by respectively 48.27 and 48.64% at rates of 5.48 and 5.82 per hour to 16.25 and 17.96% at rates of 1.5 and 1.14 per hour at the end of the incubation (Table 5.2 and Figure 5.3). With wheat straw treated with urea, Abo 374 and EFE 2 increased GP at 36 hours by respectively 9.32 and 11.68% at rates of 2.57 and 2.78 per hour to 9.6 and 13.86% at rates of 1.9 and 2.18 per hour at 48 hours (Table 5.3 and Figure

5.4). Abo 374 had the highest positive response on the GP of concentrate diet from 12 to 48 hours. At 12 hours, the increase was 1.83% at a rate 11.61 per hour compared to 9.13% at a rate of 4.72 per hour at the end of the incubation (Table 5.4 and Figure 5.5).

Table 5.3 Cumulative GP profiles, DMD, CP degradation and MPS (measured as purine derivates) on GP residues of wheat straw treated with urea.

Wheat straw treated with urea	Cumulative GP, ml/g OM												
Time, hours	Control			Abo 374			EFE 2			Yeast preparation			
1.5	1.23	±	0.31	2.62	±	0.70	0.98	±	0.41	1.04	±	0.46	
3	2.43	±	0.42	3.86	±	0.63	2.58	±	0.53	2.24	±	0.59	
6	5.23	±	0.57	7.70	±	0.54	6.61	±	0.85	5.46	±	0.65	
9	13.88	±	1.26	17.07	±	1.07	16.24	±	1.45	15.49	±	1.05	
12	28.94	±	2.10	30.94	±	1.76	31.56	±	2.08	30.61	±	1.48	
24	83.05	±	2.30	88.06	±	1.62	87.12	±	2.77	83.06	±	3.39	
36	109.81^{a}	±	2.67	120.04^{b}	±	2.08	122.63^{b}	±	4.77	113.38^{a}	±	5.84	
48	126.25^{a}	±	4.39	138.37^{b}	±	3.52	143.75^{b}	±	6.71	129.53^{a}	±	8.70	
ANOVA GP P values	Treatment 0.0027		Time <0.0001	Treatment X Time 0.2321									
Time, hours	Control			Abo 374			EFE 2			Yeast preparation			P values
MPS, µg RNA/g	73.15	±	3.91	71.91	±	5.32	73.4	±	2.25	73.36	±	2.63	0.9906
DMD, %	32.67^{a}	±	0.22	40.74^{b}	±	0.73	37.27^{c}	±	0.93	36.22^{d}	±	0.48	0.0002
CP degradat, %	37.5	±	0.81	40.77	±	1.2	39.97	±	2.19	39.08	±	0.69	0.3882

Means (± standard error mean, s.e.m) within rows with different superscript letters differ (P ≤ 0.05).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test.

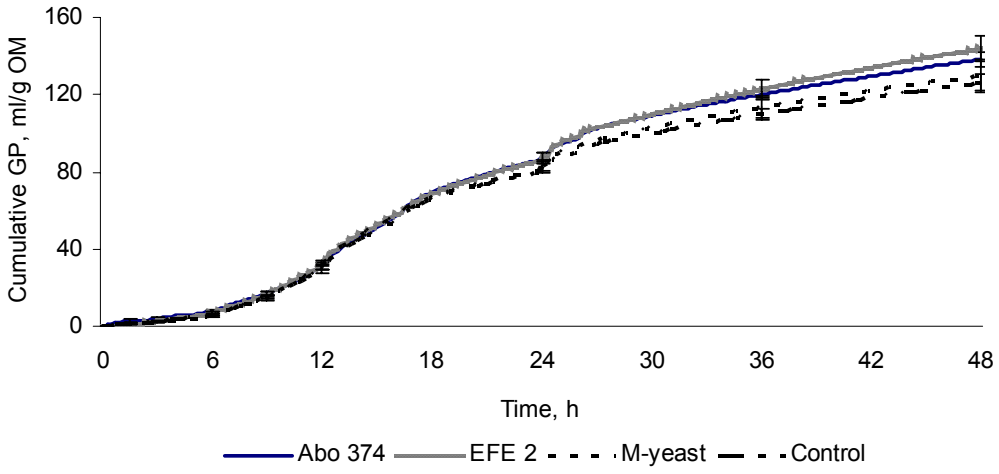

Figure 5.4 Cumulative GP profiles* of wheat straw with urea incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = - 13.78 + 4.41 time – 0.030 time^2, R^2 = 0.97; GP (Abo 374) = - 13.27 + 4.59 time – 0.028 time^2, R^2 = 0.98; GP (EFE 2) = - 14.38 + 4.58 time – 0.025 time^2, R^2 = .096; GP (Yeast prep.) = - 14.38 + 4.52 time – 0.030 time^2, R^2 = 0.94.

Table 5.4 Cumulative GP profiles, DMD, CP degradation and MPS (measured as purine derivates) on GP residues of concentrate diet.

Concentrate diet	Cumulative GP, ml/g OM											
Time, hours	Control			Abo 374			EFE 2			Yeast preparation		
1.5	21.33	±	1.01	25.57	±	1.80	24.19	±	0.74	18.20	±	2.42
3	43.06	±	2.20	48.99	±	2.49	46.05	±	1.87	39.28	±	4.53
6	105.08	±	3.18	107.40	±	5.72	102.42	±	3.87	95.70	±	7.38
9	173.91^{a}	±	3.45	170.43^{a}	±	7.82	167.02^{ab}	±	5.55	158.91^{b}	±	9.05
12	215.48^{ab}	±	5.66	219.43^{b}	±	8.67	211.53^{ab}	±	6.38	202.45^{b}	±	10.78
24	265.73^{a}	±	5.94	283.35^{b}	±	6.13	273.94^{ab}	±	5.36	266.88^{a}	±	8.54
36	296.73^{a}	±	7.24	320.45^{b}	±	5.45	308.45^{ab}	±	5.39	302.72^{a}	±	7.91
48	303.58^{a}	±	7.60	331.30^{b}	±	5.06	318.15^{c}	±	5.27	313.99^{ac}	±	7.71
ANOVA GP	Treatment		Time	Treatment X Time								
P values	<0.0001		<0.0001	0.2728								
Time, hours	Control			Abo 374			EFE 2			Yeast preparation		P values
MPS, µg RNA/g	189.6^{a}	±	10.8	242.6^{b}	±	7.47	199.9^{a}	±	6.69	192.02^{a}	±	4.97 <0.0001
DMD, %	66.8	±	2.21	70.2	±	0.38	69.19	±	1.23	69.92	±	0.84 0.3342
CP degradat., %	48.29	±	1.52	46.68	±	1.82	51.01	±	1.25	51.33	±	1.52 0.1684

Means (± standard error mean, s.e.m) within rows with different superscript letters differ (P ≤ 0.05).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test.

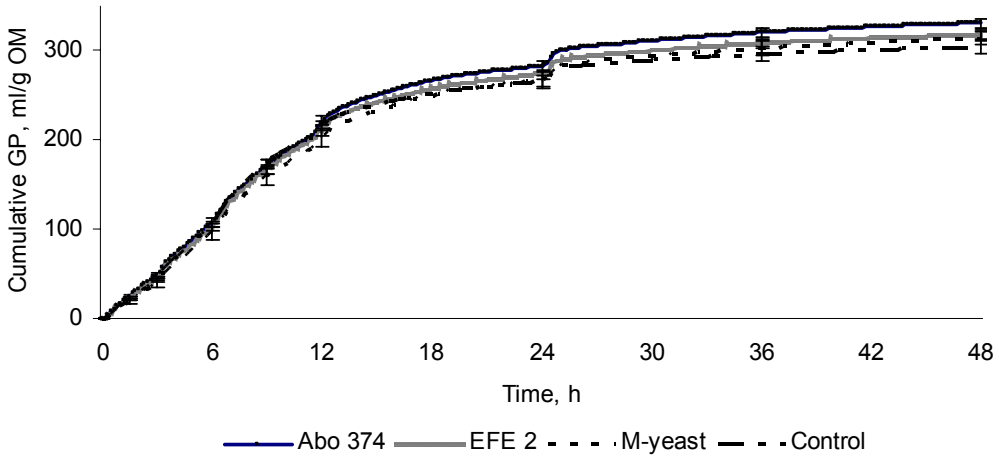

Figure 5.5 Cumulative GP profiles of concentrate diet incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 12.62 + 16.89 time – 0.23 time^2, R^2 = 0.94; GP (Abo 374) = 13.02 + 17.36 time – 0.23 time^2, R^2 = 0.97; GP (EFE 2) = 11.68 + 16.89 time – 0.22 time^2, R^2 = 0.96; GP (Yeast prep.) = 15.31 + 16.72 time – 0.22 time^2, R^2 = 0.94.

Significant effect of treatments on DM disappearance was found on wheat straw with urea when compared to other substrates. EFE (Abo 374 and EFE 2) and yeast treatments respectively improved the DM digestion of wheat straw with urea by up to 24.7, 14.16 and 10.85% in the GP system. The CP degradation of all residues of GP did not change significantly. The CP on residues of wheat straw post-incubation was two times higher than in the initial sample (39.77 g CP/kg DM). One of the greatest difficulties with CP evaluations especially with low-protein and high-fibre feedstuffs in ruminant systems is to determine the microbial contamination in

incubated residues. Microbial Nitrogen (N) can amount to as much as 95% of the residual N and microbial DM can amount to up to 22% of residual DM (Olubobokun *et al*., 1990). In fact, residues from the GP system were not washed post incubation. Vials were ice-cooled at the end of the incubation and its contents were centrifuged at 4000 rpm at 4 °C. The supernatant was decanted before the drying process. Therefore the higher CP found residues of wheat straw is probably due to microbial contamination (Vanzant *et al*., 1998).

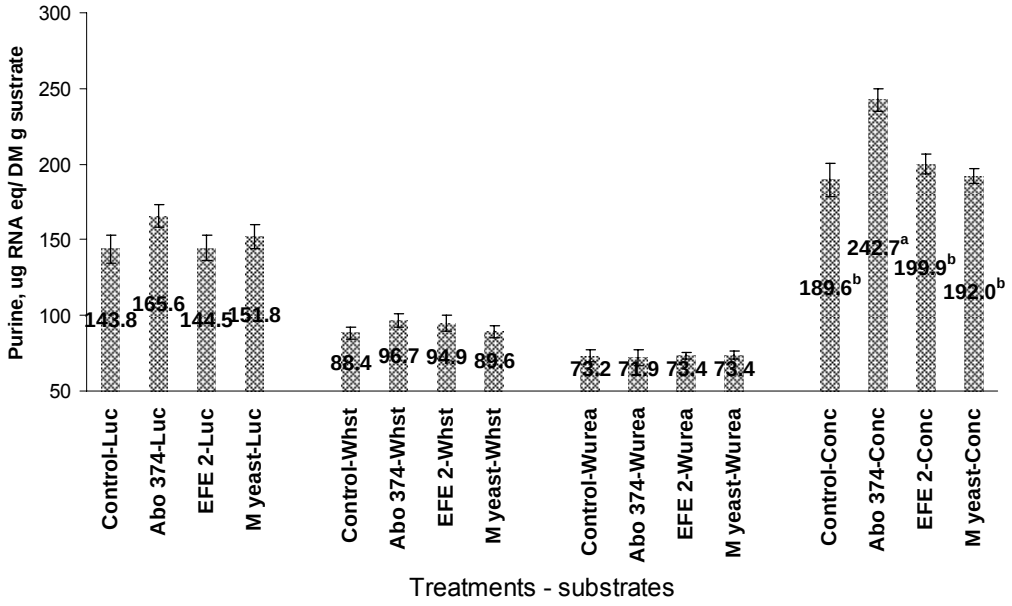

Figure 5.6 Microbial protein synthesis measured as purine derivates (µg RNA equivalent/ DM g substrate) on residues of GP of different substrates (Luc: lucerne hay, Whst: wheat straw, Wurea: wheat straw treated with urea and Conc: concentrate diet). Substrates were incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation for 48 hours. Error bars indicate the standard error of means (s.e.m).

Eun & Beauchemin (2007) evaluated the potential of different endoglucanases and xylanases with different activities using the *in vitro* GP system at 18 hours. They found that EFE on lucerne hay can improve the total GP. In another study, Kung *et al.* (2002) found that the *in vitro* GP from untreated forage was significantly lower than from forages treated with EFE. Krishnamoorthy *et al*. (1991) reported a positive linear relationship between bacterial MPS and cumulative GP (up to 8 hours of incubation) using mixed carbohydrate as substrate without EFE addition. In the current study, no significant differences have been observed on MPS measured as purine derivates done on residues of GP of lucerne hay, wheat straw and wheat straw with urea treated with EFE (Abo 374 and EFE 2) and microbial yeast (Figure 5.6). In addition, no correlation was found between MPS and the cumulative GP at 48 hours ($P < 0.05$; $R^2 < 0.30$). Lack of significance on MPS with EFE was also reported by different authors (Beauchemin *et al*., 1999; Yang *et al*., 2002; Peters *et al*., 2010). However, Abo 374 treatment increased MPS significantly on a concentrate diet by 27.99% compared to no enzyme treatment (Figure 5.6). Similarly, Yang *et al*. (1999) found that EFE improved ruminal CP degradation and bacterial protein synthesis. Consistent with this fact, Senthilkumar *et al*. (2007) reported that EFE improved GP and MPS. These authors speculated that the solubility effect of EFE on feeds possibly removed

structural barriers of digestion and released more nutrients available to support the production of bacterial glycocalyx, which improved the colonization of plant cell walls and the activity of rumen micro-organisms.

Bacterial growth curves obtained from the GP system are characterised by several phases (Cone, 1998). These phases include: time lag, exponential growth, decelerating growth, stationary and decline. During the initial hours of fermentation, the availability of rapidly fermentable components in substrate and rumen fluid inoculum generally does not limit the rate of microbial growth and the GP reaches its maximum (12 hours). This period was reported to be accompanied by a decrease in NH_3 and an increase in MPS (Cone, 1998). Subsequently the NH_3 level and the amount of MPS stayed constant until about 15 hours. Thereafter the amount of NH_3 increased and the MPS decreased. Raab (1980), as cited by Krishnamoorthy *et al*. (1991), observed that longer *in vitro* incubations reduced the microbial net growth even though the cumulative GP continued to increase. This is due to the fact that the GP, immediately after the depletion of readily fermentable components in the milieu, is the result of fermentation of intracellular glucose taken up by the micro-organisms (Cone, 1998). An uncoupled fermentation may also contribute to the reduced net growth at longer incubation times (Krishnamoorthy *et al*., 1991). However, this was not the major reason for the lack of effect on MPS with lucerne hay, wheat straw and wheat straw with urea. This may be due to an increased microbial lysis as a consequence of substrate exhaustion with 48 hours of incubation (Van Nevel & Demeyer, 1977).

The effects of EFE (Abo 374 or EFE 2) or yeast preparation on DM disappearance of different substrates are presented in Table 5.5. The DM disappearance of lucerne hay was not affected by treatment or by the interaction effects of treatment and time. At 48 hours, EFE (Abo 374 and EFE 2) and yeast preparation were respectively 6.7, 5.5 and 6.9% superior to control ($P = 0.2927$). These improvements were respectively 101.5, 101.5 and 105.6 folds compared to 91.8 folds of control relative to their zero hour (Figure 5.7). As indicated in Table 5.5, treatments and time, together with their interaction had significant effects on DM disappearance of wheat straw and wheat straw treated with urea ($P < 0.05$) (Figures 5.8-9). Abo 374 treatment was the best treatment to degrade the DM on both substrates ($P < 0.0001$). The disappearance of DM at 48 hours was 400 times higher than to its relative zero hour disappearance on both substrates with Abo 374 (460.7 folds as 24.9% on wheat straw and 440 folds as 42.4% on wheat straw treated with urea). Regardless of EFE and yeast treatment effects, treating wheat straw with urea decreased the DM disappearance by 30.6% compared to wheat straw. As mentioned by Slyter *et al.* (1979), the poor readily available energy found in wheat straw combined with high N incorporation as 2% urea to improve MPS could have created a reduced microbial synthesis due to ammonia toxicity. There were significant effects of treatments and time on the DM disappearance of the concentrate diet ($P < 0.0001$) whereas their interaction effect was not significant ($P = 0.8081$). At the end of the digestion period, EFE (Abo 374 and EFE 2) and yeast preparation significantly improved disappearance respectively by 5.1, 3.6 and 4.7% ($P < 0.0001$). The increments were respectively 127.2, 125.4 and 123.6 folds relative to their zero hour disappearance compared to 116.9 folds of control (Figure 5.10).

Table 5.5 Effects of EFE and microbial yeast preparation on the *in vitro* DM disappearance of different substrates (*in vitro* filter bag technique).

Lucerne: DM disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	33.50	±	0.62	34.12	±	0.44	33.65	±	0.15	33.44	±	0.21
3	38.47	±	0.38	38.78	±	0.44	37.78	±	0.36	37.52	±	0.79
6	49.12	±	1.32	48.08	±	1.34	46.15	±	0.63	43.59	±	0.87
9	57.09	±	1.80	55.32	±	1.22	57.04	±	0.60	55.43	±	1.28
12	59.08	±	2.31	60.22	±	1.45	59.74	±	0.89	59.82	±	1.28
24	60.99^{a}	±	2.08	65.23^{b}	±	0.87	64.34^{ab}	±	1.23	63.97^{b}	±	0.30
48	64.26^{a}	±	1.97	68.61^{b}	±	0.39	67.82^{b}	±	0.97	68.73^{b}	±	0.80
ANOVA DM P values	Treatment 0.2927		Time <0.0001	Treatment X Time 0.2521								
Wheat straw: DM disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	6.99	±	0.28	7.25	±	0.46	7.84	±	0.33	7.22	±	0.33
3	8.39^{a}	±	0.23	9.38^{b}	±	0.30	9.65^{b}	±	0.22	9.20^{ab}	±	0.39
6	10.46	±	0.28	10.63	±	0.16	11.11	±	0.17	10.24	±	0.34
9	12.38^{a}	±	0.46	12.46^{a}	±	0.17	13.41^{b}	±	0.24	13.19^{ab}	±	0.33
12	15.61^{a}	±	0.38	17.48^{b}	±	0.16	16.24^{a}	±	0.58	15.75^{a}	±	0.50
24	22.13^{a}	±	0.17	26.69^{b}	±	0.30	24.71^{c}	±	0.63	26.52^{b}	±	0.39
48	32.53^{a}	±	0.20	40.63^{b}	±	0.44	38.35^{c}	±	0.26	36.08^{d}	±	0.41
ANOVA DM P values	Treatment <.0001		Time <0.0001	Treatment X Time <.0001								
Wheat straw treated with urea: DM disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	6.13	±	0.56	6.41	±	0.38	6.78	±	0.34	6.89	±	0.38
3	7.89	±	0.42	7.82	±	0.25	8.62	±	0.56	8.57	±	0.24
6	9.13	±	0.25	9.26	±	0.48	9.65	±	0.61	9.47	±	0.56
9	10.74	±	0.53	10.71	±	0.60	10.90	±	0.59	11.42	±	0.57
12	11.94	±	0.44	14.15	±	0.78	12.59	±	0.65	14.18	±	0.72
24	17.68^{a}	±	0.86	25.68^{b}	±	0.97	18.79^{a}	±	1.23	19.35^{a}	±	0.87
48	24.29^{a}	±	1.48	34.60^{b}	±	1.52	28.06^{c}	±	1.86	26.06^{ac}	±	1.40
ANOVA DM P values	Treatment <.0001		Time <0.0001	Treatment X Time <.0001								
Concentrate diet: DM disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	30.75	±	0.69	30.81	±	0.56	30.65	±	0.59	31.23	±	0.48
3	33.79	±	0.59	35.19	±	0.69	33.62	±	0.91	34.96	±	0.78
6	37.14	±	0.71	`38.32	±	0.92	36.54	±	0.89	37.00	±	0.43
9	41.30	±	1.05	42.94	±	0.56	40.74	±	1.19	44.38	±	0.85
12	47.53^{a}	±	1.64	52.97^{c}	±	1.09	48.73^{ab}	±	0.65	50.45^{cb}	±	1.81
24	58.67^{a}	±	1.42	62.21^{b}	±	1.31	59.55^{ab}	±	1.35	60.90^{ab}	±	1.71
48	66.69^{a}	±	1.15	70.09^{b}	±	0.50	69.09^{ab}	±	0.92	69.83^{b}	±	0.84
ANOVA DM P values	Treatment 0.0001		Time <0.0001	Treatment X Time 0.8081								

Means (± standard error mean, s.e.m) within rows with different superscript letters differ (P ≤ 0.05).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test.

The effects of EFE (Abo 374 or EFE 2) or yeast preparations on the disappearance of the NDF fraction of different substrates are presented in Table 5.6 and Figures 5.11-14. Treatment effects were significant on NDF disappearance of lucerne hay (P < 0.0001), with Abo 374 being the best treatment. Improvement of

3.91% was observed with Abo 374 enzyme at 48 hours of incubation. No effects of treatments and time as well as their interactions were found significant on NDF disappearance of wheat straw, wheat straw treated with urea and concentrate diet.

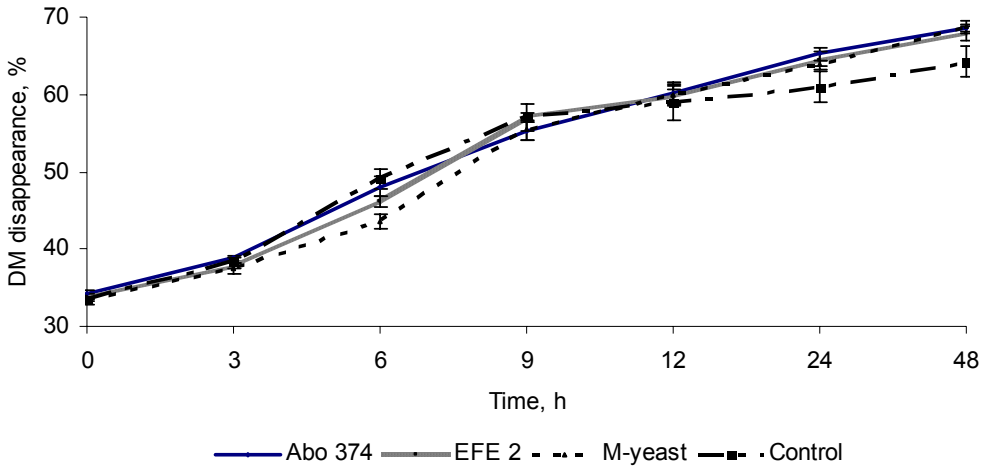

Figure 5.7 Dry matter disappearance* of lucerne hay incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 36.10 + 2 time – 0.03 time^2, R^2 = 0.75; GP (Abo 374) = 35.31 + 2.16 time – 0.031 time^2, R^2 = 0.91; GP (EFE 2) = 34.99 + 2.15 time – 0.03 time^2, R^2 = 0.91; GP (Yeast prep.) = 34.51 + 2.13 time – 0.03 time^2, R^2 = 0.91.

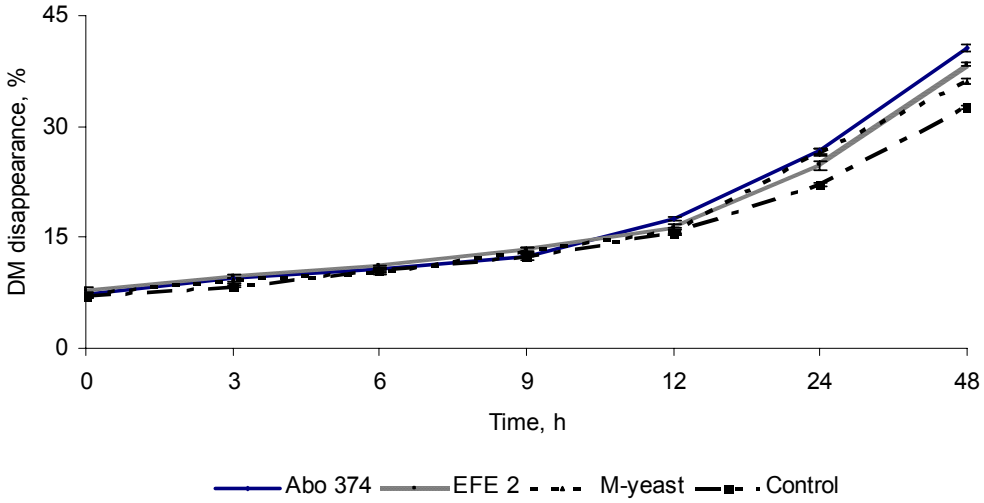

Figure 5.8 Dry matter disappearance* of wheat straw incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 6.46 + 0.76 time – 0.0045 time^2, R^2 = 0.99; GP (Abo 374) = 6.31 + 0.91 time – 0.004 time^2, R^2 = 0.97; GP (EFE 2) = 7.39 + 0.77 time – 0.0025 time^2, R^2 = 0.99; GP (Yeast prep.) = 6.08 + 0.94 time – 0.0066 time^2, R^2 = 0.98.

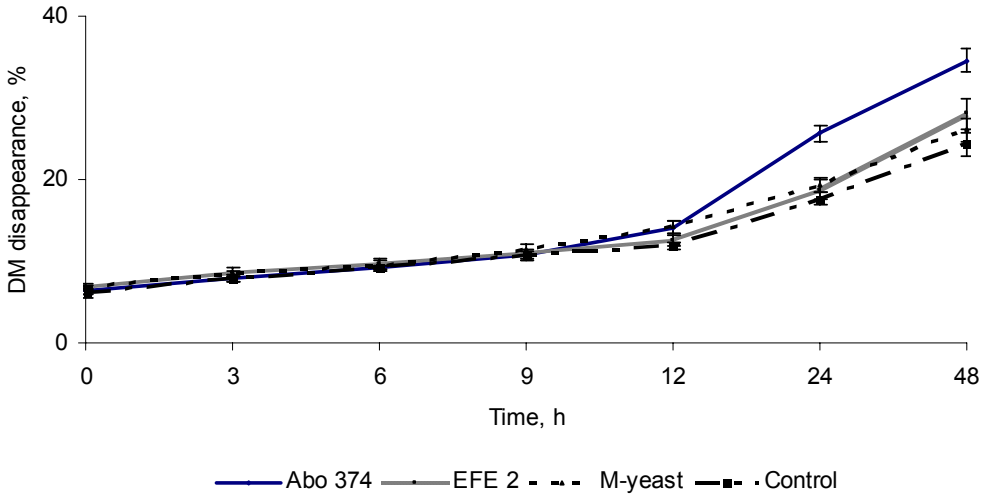

Figure 5.9 Dry matter disappearance* of wheat straw treated with urea incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 6.04 + 0.56 time – 0.0038 time^2, R^2 = 0.88; GP (Abo 374) = 4.77 + 0.93 time – 0.0063 time^2, R^2 = 0.93; GP (EFE 2) = 6.72 + 0.53 time – 0.0018 time^2, R^2 = 0.86; GP (Yeast prep.) = 6.65 + 0.64 time – 0.0050 time^2, R^2 = 0.89.

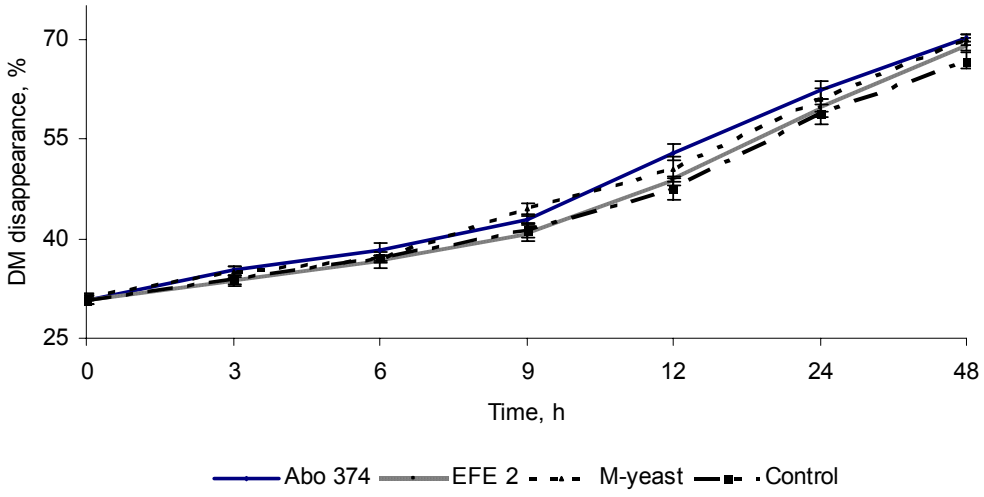

Figure 5.10 Dry matter disappearance* of the concentrate diet incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 29.44 + 1.61 time – 0.017 time^2, R^2 = 0.93; GP (Abo 374) = 29.7 + 1.91 time – 0.022 time^2, R^2 = 0.95; GP (EFE 2) = 29.35 + 1.69 time – 0.018 time^2, R^2 = 0.95; GP (Yeast prep.) = 19.99 + 1.74 time – 0.019 time^2, R^2 = 0.93.

Table 5.6 Effects of EFE and microbial yeast preparation on the *in vitro* NDF disappearance of different substrates (*in vitro* filter bag technique).

Lucerne: NDF disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	60.96	±	0.21	61.53	±	0.73	59.74	±	0.82	60.03	±	0.76
3	61.26	±	0.58	63.59	±	0.45	61.91	±	1.37	61.49	±	0.65
6	62.19[a]	±	0.44	65.69[b]	±	0.80	64.05[ab]	±	0.89	63.12[a]	±	0.72
9	66.65	±	1.18	67.96	±	0.79	66.51	±	0.92	66.09	±	0.40
12	69.49	±	1.02	69.97	±	0.95	68.28	±	1.18	68.05	±	0.38
24	70.96[a]	±	0.57	73.64[b]	±	0.43	71.11[ab]	±	1.18	70.93[a]	±	0.46
48	73.08[ab]	±	0.38	75.94[b]	±	0.46	73.44[ab]	±	1.51	72.66[a]	±	1.08
ANOVA NDF P values	Treatment <.0001		Time <0.0001	Treatment X Time 0.941								
Wheat straw: NDF disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	15.66	±	1.17	14.86	±	1.39	15.33	±	1.17	15.68	±	1.60
3	16.82	±	1.35	16.97	±	1.18	16.56	±	1.61	16.68	±	2.32
6	18.50	±	1.22	18.92	±	1.32	17.75	±	1.27	18.11	±	2.60
9	21.84	±	1.13	22.33	±	0.91	20.63	±	0.73	21.72	±	2.38
12	25.69	±	2.56	27.20	±	0.67	23.80	±	1.67	22.83	±	1.02
24	28.34	±	1.75	32.18	±	1.19	29.07	±	1.88	28.51	±	1.27
48	34.32	±	1.05	37.70	±	1.48	35.20	±	2.04	34.81	±	1.07
ANOVA NDF P values	Treatment 0.2298		Time <0.0001	Treatment X Time 0.9844								
Wheat straw treated with urea: NDF disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	15.91	±	1.34	16.46	±	0.35	16.83	±	0.39	16.58	±	0.56
3	17.91	±	1.66	17.65	±	0.04	17.71	±	0.52	17.67	±	0.92
6	19.27	±	1.62	19.33	±	0.38	19.29	±	0.38	19.03	±	0.87
9	21.83	±	1.75	20.73	±	0.86	21.49	±	0.47	21.79	±	0.60
12	24.48	±	1.81	23.37	±	0.48	24.51	±	0.55	24.15	±	0.96
24	29.76	±	1.01	29.64	±	0.87	29.45	±	1.03	29.80	±	1.18
48	35.86	±	0.84	39.00	±	2.52	36.75	±	0.59	35.64	±	1.18
ANOVA NDF P values	Treatment 0.9602		Time <0.0001	Treatment X Time 0.9503								
Concentrate diet: NDF disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	59.82	±	2.86	59.80	±	2.41	63.45	±	0.75	62.47	±	1.54
3	62.91	±	1.59	61.76	±	1.79	63.41	±	1.67	61.91	±	1.30
6	61.85	±	0.81	63.37	±	2.16	65.88	±	1.37	62.66	±	1.27
9	67.79	±	0.90	65.01	±	1.72	67.98	±	1.73	65.70	±	2.20
12	68.74	±	0.54	69.17	±	3.31	68.40	±	2.05	70.36	±	1.88
24	71.77	±	1.16	73.87	±	2.25	72.33	±	0.66	73.46	±	0.66
48	73.03	±	0.90	76.10	±	0.99	73.95	±	1.47	72.45	±	1.89
ANOVA NDF P values	Treatment 0.6288		Time <0.0001	Treatment X Time 0.8261								

Means (± standard error mean, s.e.m) within rows with different superscript letters differ ($P \leq 0.05$).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test.

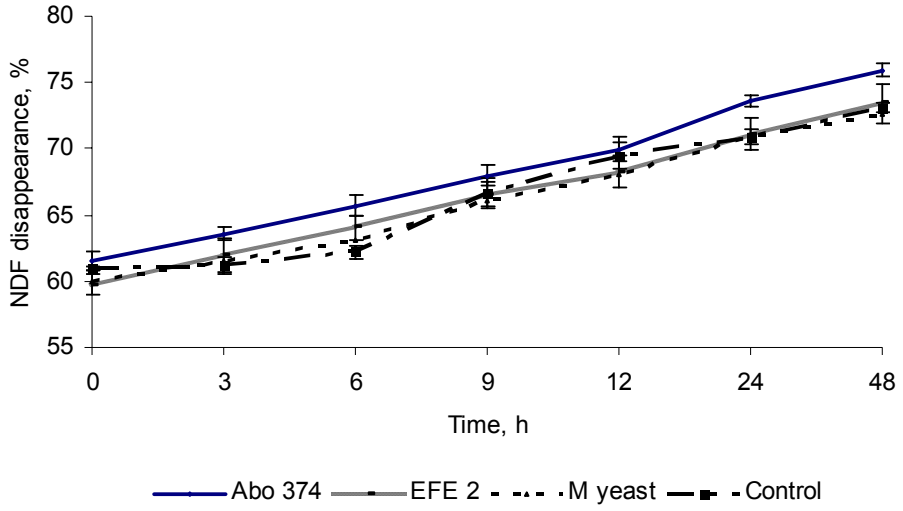

Figure 5.11 Neutral-detergent fibre disappearance* of lucerne hay incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 60.13 + 0.72 time – 0.0094 time^2, R^2 = 0.88; GP (Abo 374) = 61.65 + 0.75 time – 0.0095 time^2, R^2 = 0.95; GP (EFE 2) = 59.92 + 0.74 time – 0.0095 time^2, R^2 = 0.85; GP (Yeast prep.) = 59.87 + 0.71 time – 0.0093 time^2, R^2 = 0.94.

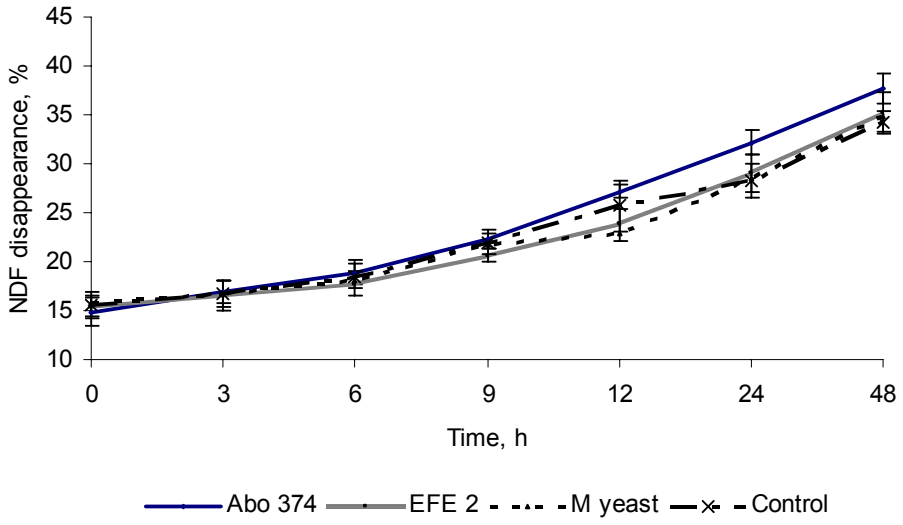

Figure 5.12 Neutral-detergent fibre disappearance* of wheat straw incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 15.23 + 0.76 time – 0.0077 time^2, R^2 = 0.89; GP (Abo 374) = 14.26 + 0.75 time – 0.072 time^2, R^2 = 0.94; GP (EFE 2) = 15.28 + 0.79 time – 0.0072 time^2, R^2 = 0.87; GP (Yeast prep.) = 15.07 + 0.71 time – 0.0062 time^2, R^2 = 0.85.

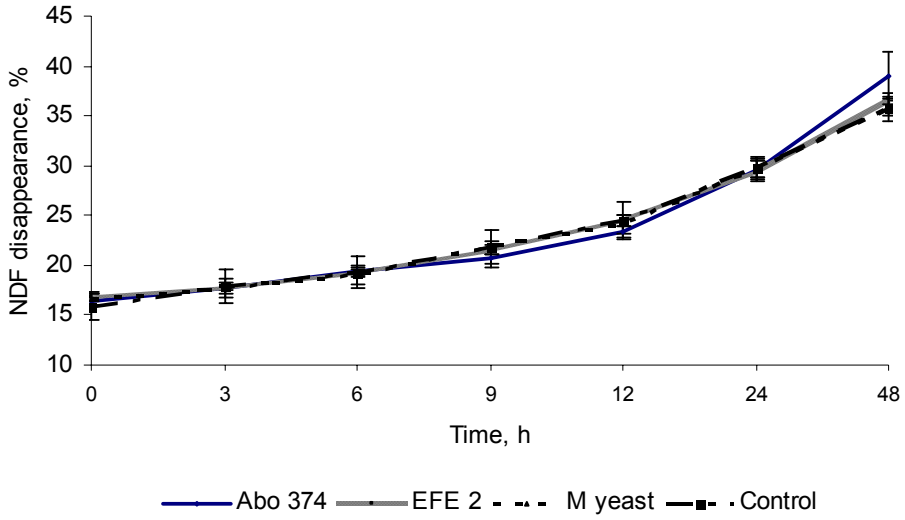

Figure 5.13 Neutral-detergent fibre disappearance* of wheat straw with urea incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 15.65 + 0.76 time – 0.0072 time^2, R^2 = 0.91; GP (Abo 374) = 15.93 + 0.63 time – 0.0031 time^2, R^2 = 0.96; GP (EFE 2) = 16.01 + 0.63 time – 0.004 time^2, R^2 = 0.99; GP (Yeast prep.) = 15.65 + 0.76 time – 0.0072 time^2, R^2 = 0.91.

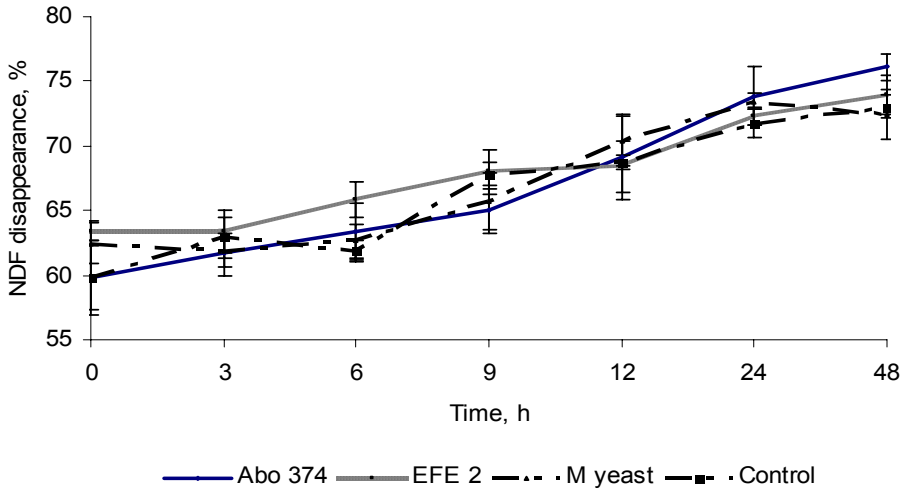

Figure 5.14 Neutral-detergent fibre disappearance* of the concentrate diet incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 59.98 + 0.78 time – 0.011 time^2, R^2 = 0.79; GP (Abo 374) = 59.26 + 0.86 time – 0.011 time^2, R^2 = 0.76; GP (EFE 2) = 62.51 + 0.53 time – 0.011 time^2, R^2 = 0.78; GP (Yeast prep.) = 60.49 + 0.80 time – 0.011 time^2, R^2 = 0.73.

The effects of EFE and yeast preparation increased the DM disappearance. Giraldo *et al*. (2007) reported that EFE stimulated the initial phases of substrate degradation, but the effects were reduced as incubation time prolonged (96 hours). Consistent with this hypothesis, Nsereko *et al*. (2002) observed that after treating

the diet of dairy cows with EFE from *T. longibrachiatum,* the number of rumen bacteria using either hemicellulose or secondary products from cellulose, can be increased. This occurs particularly during the early phase of digestion. Dawson & Tricarico (1999) speculated that EFE may act in the rumen shortly after feeding through enhancement of microbial colonization and synergy with endogenous enzymes. In contrast to these studies, Lewis *et al*. (1996) found no effect of EFE on *in situ* disappearance of DM, NDF and acid-detergent fibre (ADF) during the initial phase of digestion, but EFE increased DM and NDF disappearance after 32, 40 and 96 hours of incubation. They speculated that the increase of DM and NDF disappearance after a long period of digestion could result from improved colonization and digestion of the slowly degradable fibre fraction by ruminal microbes. Feng *et al.* (1996) also reported higher *in situ* DM disappearance with EFE treated grass substrate after 24 and 48 hours. Tang *et al*. (2008) evaluated the *in vitro* effects of EFE and yeast preparation on rice straw, wheat straw, maize stover and ensiled maize stover. Both EFE and yeast preparations were significantly able to increase the GP and disappearance of DM and NDF in all low-quality cereal straws. Guedes *et al*. (2008) also reported that feeding 1.0 g/day of microbial yeast preparation (1×10^{10} CFU/g) from *Saccharomyces cerevisiae* has the potential to increase rumen fibrolytic activity and NDF degradation by alleviating pH depression after feeding. In another investigation, the microbial yeast preparation was found to improve the rumen microbial activity in young ruminants, stabilisation of rumen pH and prevention of acidosis in dairy cows. The enhancement of feed digestion was reported with the yeast preparation as a result of: (1) the improvement of rumen maturity by favouring microbial establishment, (2) the stabilisation of ruminal pH with suppression of lactate-metabolising bacteria and (3) the increase of fibre degradation and interactions with plant–cell wall degrading microorganisms (Chaucheyras-Durand *et al*., 2008). Contrary to these results, there are some studies which indicated that the use of EFE did not result in significant increase in the digestion of fibrous diets in ruminants (Hristov *et al*. 2000; Bowman *et al*., 2003). Similar to these finding, this study revealed that EFE were inconsistent to significantly improve the NDF disappearance of wheat straw, wheat straw treated with urea and concentrate diet. González-García *et al*. (2010) found no improvement of NDF disappearance with EFE addition in high fibre diets but increased GP. No effects of EFE were also found on *in vitro* DM and NDF degradation as well as on GP of both concentrate diets and forage hays (Baloyi, 2008).

The effects of EFE (Abo 374 or EFE 2) or yeast preparations on the CP degradation of different substrates are presented in Table 5.7. The CP of wheat straw and concentrate diet was observed to be degraded significantly higher by treatments ($P < 0.05$) whereas treatment effects did not differ for CP degradation of lucerne hay and wheat straw treated with urea. The time had a significant effect whereas the interaction effect of treatment and time was not significant on all four tested substrates. The EFE and microbe yeast significantly improved CP disappearance of wheat straw and concentrate diet (Figure 5.16 and 5.18). The lack of significance on CP disappearance was also reported by Yang *et al*. (2002) as in this study with lucerne hay and wheat straw with urea. In another study, adding EFE product to a total mixed diet right before feeding improved ruminal fibre digestion, but did not affect ruminal N metabolism in dairy cows (Beauchemin *et al*., 1999). As pointed out, the microbial contamination with incubated residues of low-protein and high-fibre feedstuffs remain a great concern when evaluating CP degradation although procedures oblige Dacron bags to be machine-rinsed for 5 minutes (Vanzant *et al*., 1998). Prior to rinsing, microbial contamination can

amount to as much as 95% of the residual N and up to 22% of residual DM (Olubobokun *et al*., 1990). The relatively high coefficients (s.e.m) of variation observed with CP degradation profiles of different substrates may be due to microbial contamination during the *in vitro* digestion.

Table 5.7 Effects of EFE and microbial yeast preparation on the *in vitro* CP disappearance of different substrates (*in vitro* filter bag technique).

Lucerne: CP disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	43.78	±	2.23	48.51	±	0.65	47.29	±	1.08	46.00	±	0.94
3	52.10	±	0.65	53.41	±	0.92	51.02	±	1.29	52.88	±	0.96
6	63.63	±	1.73	60.56	±	0.65	61.33	±	1.66	56.98	±	2.65
9	74.75	±	2.97	70.67	±	1.65	72.91	±	1.50	69.45	±	2.22
12	75.42	±	3.78	76.30	±	2.23	77.43	±	1.53	78.18	±	2.02
24	78.75	±	2.86	83.05	±	0.33	79.87	±	2.61	83.19	±	0.49
48	83.16	±	1.47	85.66	±	0.92	85.40	±	0.50	85.86	±	0.65
ANOVA CP P values	Treatment 0.7554		Time <0.0001	Treatment X Time 0.183								
Wheat straw: CP disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	18.66	±	1.15	20.30	±	1.01	20.22	±	0.70	21.80	±	0.84
3	19.50^{a}	±	1.00	20.45^{a}	±	1.50	21.73^{a}	±	1.04	26.67^{b}	±	1.40
6	22.39^{a}	±	1.32	22.53^{a}	±	1.21	23.03^{a}	±	1.29	27.18^{b}	±	1.64
9	23.72^{a}	±	1.64	22.33^{a}	±	0.80	27.62^{a}	±	3.04	29.20^{b}	±	1.26
12	25.40^{a}	±	1.47	29.96^{c}	±	2.46	26.31^{ab}	±	1.48	29.46^{bc}	±	1.35
24	30.67	±	1.90	32.17	±	1.55	30.79	±	1.24	31.67	±	1.21
48	30.59^{a}	±	4.18	40.52^{b}	±	1.22	36.56^{a}	±	1.09	33.00^{a}	±	3.05
ANOVA CP P values	Treatment <0.0001		Time <0.0001	Treatment X Time 0.0025								
Wheat straw treated with urea: CP disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	49.97	±	0.55	49.27	±	0.83	49.06	±	0.73	49.58	±	0.58
3	49.15	±	1.57	49.56	±	0.82	49.17	±	0.88	50.46	±	0.35
6	49.98	±	0.57	50.17	±	0.69	49.81	±	1.08	49.60	±	0.62
9	51.40	±	0.49	50.14	±	0.78	51.22	±	0.94	50.85	±	0.48
12	52.00	±	0.54	51.33	±	0.61	52.52	±	0.81	51.64	±	0.33
24	54.34	±	0.70	54.82	±	0.57	54.16	±	0.76	55.97	±	0.42
48	57.78	±	0.48	58.89	±	0.88	58.00	±	1.06	58.56	±	0.69
ANOVA CP P values	Treatment 0.7535		Time <0.0001	Treatment X Time 0.9117								
Concentrate diet: CP disappearance, %												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
0	46.63	±	1.52	48.90	±	0.90	47.81	±	0.85	46.35	±	1.37
3	49.60	±	1.89	53.48	±	1.12	50.51	±	2.61	52.10	±	2.37
6	50.09	±	1.02	50.72	±	1.61	49.31	±	0.89	50.30	±	1.71
9	49.82	±	0.98	51.61	±	1.68	51.35	±	1.58	54.51	±	0.93
12	50.38^{a}	±	3.09	60.83^{c}	±	1.00	54.09^{ab}	±	1.58	59.16^{bc}	±	1.60
24	60.14	±	6.55	65.76	±	2.33	62.57	±	4.22	67.04	±	3.02
48	71.26^{a}	±	4.60	79.19^{b}	±	0.81	75.69^{ab}	±	2.64	75.71^{ab}	±	1.82
ANOVA CP P values	Treatment 0.0022		Time <0.0001	Treatment X Time 0.8853								

Means (± standard error mean, s.e.m) within rows with different superscript letters differ (P ≤ 0.05).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test.

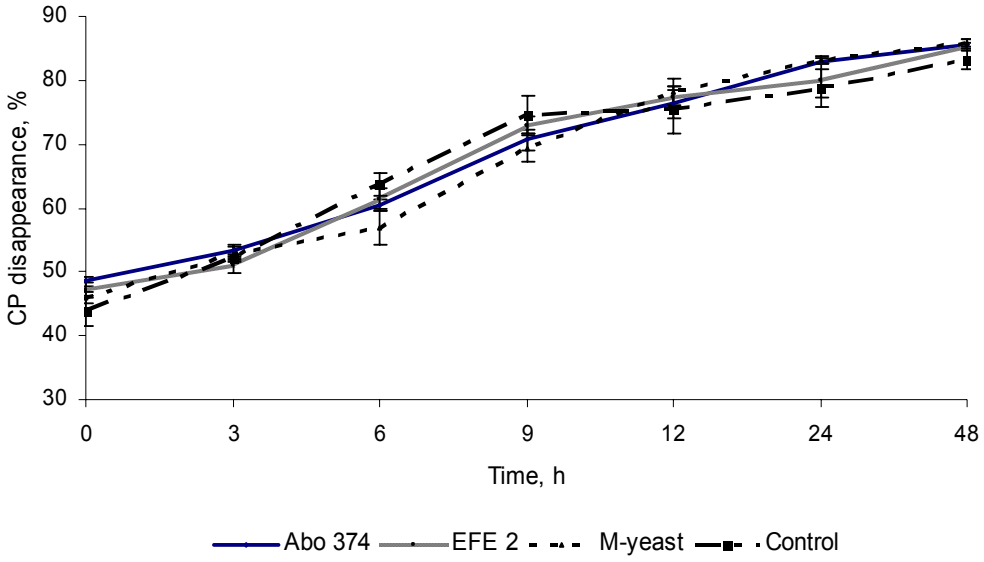

Figure 5.15 Crude protein disappearance* of lucerne hay incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 47.91 + 2.47 time – 0.037 time^2, R^2 = 0.76; GP (Abo 374) = 48.76 + 2.4 time – 0.034 time^2, R^2 = 0.93; GP (EFE 2) = 48.45 + 2.39 time – 0.034 time^2, R^2 = 0.85; GP (Yeast prep.) = 46.34 + 2.61 time – 0.038 time^2, R^2 = 0.89.

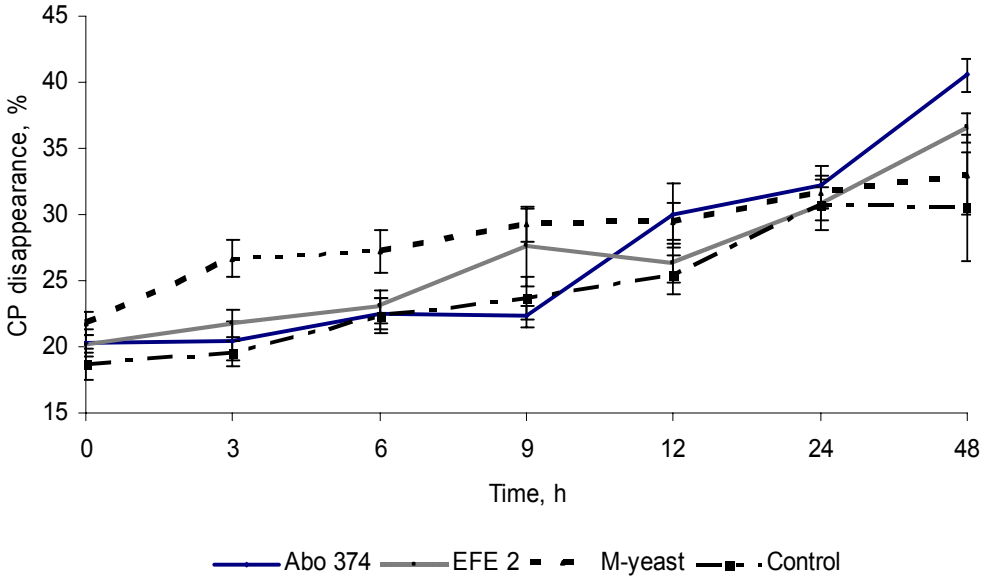

Figure 5.16 Crude protein disappearance* of wheat straw incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 18.21 + 0.69 time – 0.0073 time^2, R^2 = 0.71; GP (Abo 374) = 19.74 + 0.64 time – 0.0042 time^2, R^2 = 0.78; GP (EFE 2) = 20.13 + 0.55 time – 0.0043 time^2, R^2 = 0.81; GP (Yeast prep.) = 24.11 + 0.51 time – 0.0057 time^2, R^2 = 0.63.

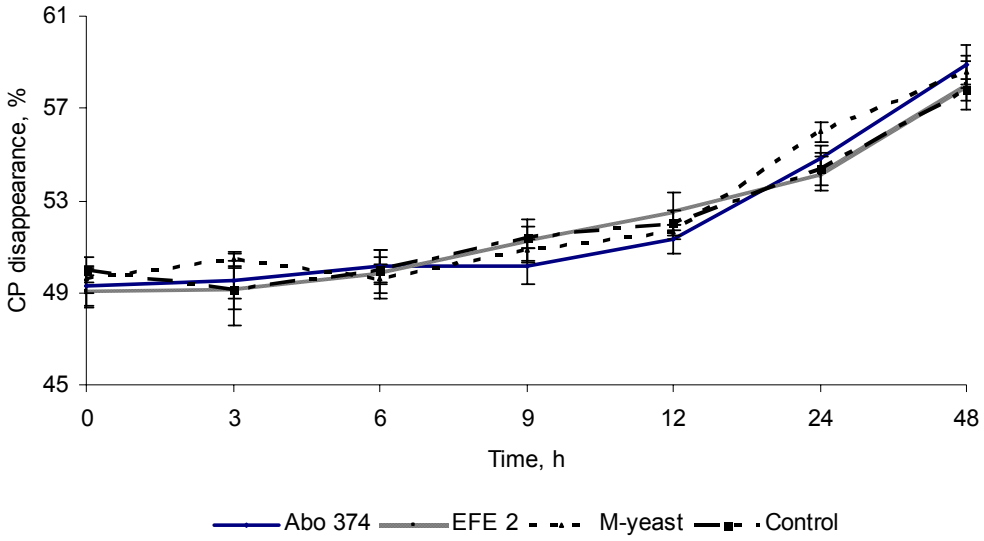

Figure 5.17 Crude protein disappearance* of wheat straw with urea incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 49.17 + 0.24 time – 0.0012 time^2, R^2 = 0.70; GP (Abo 374) = 48.81 + 0.24 time – 0.00048 time^2, R^2 = 0.78; GP (EFE 2) = 48.71 + 0.28 time – 0.0018 time^2, R^2 = 0.67; GP (Yeast prep.) = 48.94 + 0.29 time – 0.0018 time^2, R^2 = 0.84.

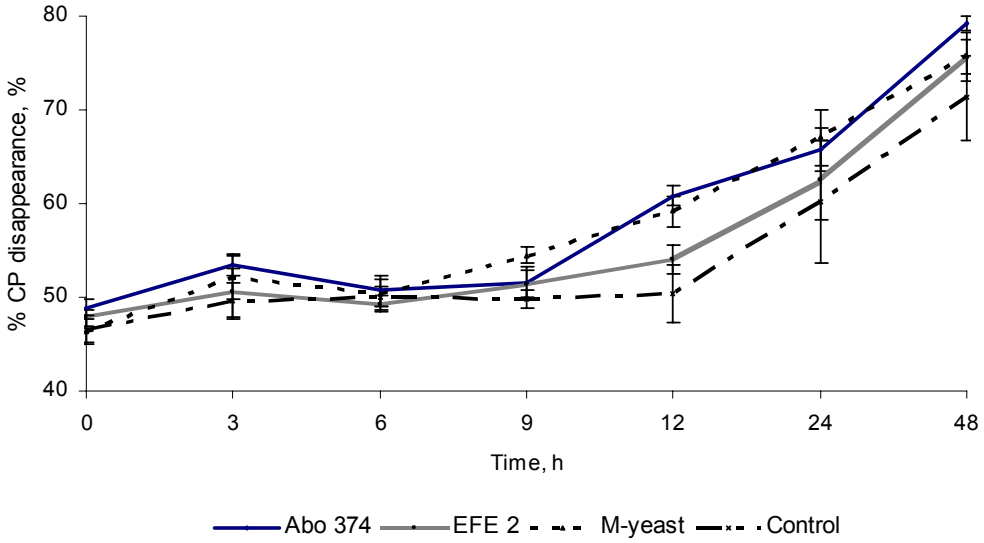

Figure 5.18 Crude protein disappearance* of concentrate diet incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: GP (Control) = 46.78 + 0.46 time + 0.0012 time^2, R^2 = 0.71; GP (Abo 374) = 48.7 + 0.76 time – 0.0026 time^2, R^2 = 0.91; GP (EFE 2) = 46.48 + 0.58 time + 0.0006 time^2, R^2 = 0.86; GP (Yeast prep.) = 46.62 + 1.07 time – 0.0097 time^2, R^2 = 0.90.

Yeast preparation significantly increased CP degradation of wheat straw from 3 to 12 hours ($P < 0.0001$). This was 36.76, 21.39, 23.10 and 17.95% higher than the control respectively at 3, 6, 9 and 12 hours of

incubation. Abo 374 significantly increased CP degradation of wheat straw by 17.95% at 12 hours and 32.4% at 48 hours of digestion (Figure 5.16). With the concentrate diet, Abo 374 and yeast treatments were the best at 12 hours of incubation (P = 0.0022) with improvements up to 20.75 and 17.43% CP disappeared respectively compared to control. At the end of the incubation, Abo 374 was 11.12% higher than the no enzyme treatment (Figure 5.18). These results were in agreement with the observation of Yang *et al.* (1999) who found that the addition of EFE enhanced ruminal CP degradation. Consistent to that hypothesis, Álvarez *et al.* (2009) found that EFE added to the diet of lactating dairy cows increased solubility of DM and CP fractions and ruminal disappearance of fibrous fractions of wheat middlings and oat straw. The authors attributed the higher total disappearance of CP to the net effect of EFE. They stipulated that EFE activities are not only limited to plant cell wall components. This would explain why EFE can be effective in improving digestibility of the non-fibre carbohydrate fraction, in addition to increasing the digestibility of the fibre components of diets (Beauchemin *et al*., 2003). In another study, the Nitrogen (N) intake, faecal N and N retention of lucerne and ryegrass hays were increased with EFE addition, therefore increasing apparent digestibility of CP (Pinos-Rodríguez *et al*., 2002). According to McAllister *et al*. (2001), EFE seem to contain some proteolytic activities as they facilitate degradation of cell wall bound proteins. In addition, EFE with cellulases as major activity was also found to increase CP degradation of forages *in vitro* by making proteins more available to proteolytic enzymes (Kohn & Allen, 1992). In agreement with this, Rode *et al.* (1999) found that EFE did not enhance the DM intake, but increased the milk production as a result of an increased digestion of energy (OM and NDF) and CP.

The effects of EFE (Abo 374 or EFE 2) or yeast preparation on bacterial protein synthesis (purine derivates) of different substrates are presented in Table 5.8. The MPS of all four tested substrates were found to be affected significantly by treatments and time (P <0.05). In general, EFE improved the bacterial protein synthesis, with a peak being between 6 and 24 hours of incubation, depending on substrate difference (Figures 5.19-22). The MPS, which tended to decrease at the end of incubation, was consistent with observations of Van Nevel & Demeyer (1977). Different responses in purine derivates may be due to differences in fermentation pathways or to differences in energetic efficiency of the structural and non-structural carbohydrates of substrates (Krishnamoorthy *et al*., 1991). The interaction effects of treatment and time were not significant on MPS of wheat straw. However these were significant on MPS of lucerne hay, wheat straw treated with urea and concentrate. These results were not in agreement with the observation of Beauchemin *et al.* (1999) who reported that adding an EFE product to a total mixed diet before feeding improved ruminal fibre digestion but did not affect ruminal N metabolism in dairy cows. Similary, Yang *et al*. (2002) and Peters *et al*. (2010) reported that *in vitro* degradation of CP and bacterial protein synthesis were not affected by adding EFE to the diet. However, the addition of EFE enhanced the ruminal CP degradation and bacterial protein synthesis in other studies (Yang *et al*., 1999). Consistent with this, Bala *et al*. (2009) observed that the milk yield of lactating goats was increased as a result of the improvement of the energy availability and the utilization of microbial digestible protein, estimated based on purine derivatives and creatinine excreted in urine. These authors speculated that EFE were able to free the trapped nutrients in the cell wall networks of roughages. The EFE was found to improve the fermentative end products as a result of the change the non glucogenic/glucogenic ratio in the rumen (Bala *et al*., 2009).

Table 5.8 Effects of EFE and microbial yeast preparation on the *in vitro* MPS measured as purine derivates of different substrates (*in vitro* filter bag technique).

Lucerne: Purine derivates, µg RNA equivalent/g DM												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
3	37.77	±	2.95	44.40	±	4.49	29.96	±	2.46	41.82	±	3.38
6	52.84	±	6.90	66.78	±	10.61	52.56	±	7.53	55.44	±	3.28
9	56.53^{a}	±	5.27	78.95^{b}	±	14.56	78.68^{b}	±	8.23	68.07^{ab}	±	9.20
12	50.24^{a}	±	3.36	90.73^{b}	±	9.67	48.52^{a}	±	4.64	49.70^{a}	±	4.12
24	39.78^{a}	±	2.72	59.63^{b}	±	8.71	32.57^{a}	±	3.45	45.78^{ab}	±	3.67
48	37.01	±	3.21	35.05	±	3.24	26.76	±	3.26	38.78	±	3.46
ANOVA Pur. P values	Treatment 0.0002		Time <0.0001	Treatment X Time 0.004								
Wheat straw: Purine derivates, µg RNA equivalent/g DM												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
3	30.21	±	5.24	36.15	±	8.19	22.35	±	4.72	21.26	±	1.75
6	45.36^{a}	±	9.84	48.93^{b}	±	7.27	38.17^{ab}	±	13.51	25.19^{a}	±	2.98
9	38.00	±	6.67	36.43	±	6.10	35.75	±	9.16	43.68	±	7.05
12	56.59^{a}	±	10.62	39.15^{ab}	±	5.23	30.61^{b}	±	5.01	51.09^{a}	±	10.45
24	34.05^{ab}	±	6.91	38.96^{a}	±	6.56	20.45^{b}	±	3.07	26.24^{ab}	±	2.21
48	27.91	±	6.00	17.56	±	2.42	19.49	±	3.14	19.47	±	4.24
ANOVA Pur. P values	Treatment 0.0351		Time 0.0001	Treatment X Time 0.2191								
Wheat straw treated with urea: Purine derivates, µg RNA equivalent/g DM												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
3	38.14	±	3.11	34.40	±	8.86	32.54	±	4.60	24.67	±	2.79
6	26.62^{a}	±	2.70	58.16^{b}	±	6.90	25.68^{a}	±	3.63	26.99^{a}	±	2.43
9	26.88a	±	3.66	46.07^{b}	±	9.63	47.93^{b}	±	7.37	44.78^{b}	±	7.90
12	26.13^{a}	±	2.75	38.80^{ab}	±	6.89	48.44^{b}	±	7.33	30.16^{a}	±	3.57
24	31.87^{ab}	±	3.37	39.69^{b}	±	4.61	32.61^{ab}	±	6.38	21.62^{a}	±	3.01
48	26.85	±	5.30	28.68	±	3.53	34.66	±	6.06	28.97	±	3.34
ANOVA Pur. P values	Treatment 0.0019		Time 0.0381	Treatment X Time 0.0013								
Concentrate diet: Purine derivates, µg RNA equivalent/g DM												
Time, hours	Control			Abo 374			EFE 2			Microbial yeast		
3	51.20^{a}	±	10.48	30.97^{b}	±	10.94	25.12^{c}	±	2.50	62.84^{a}	±	15.48
6	40.47	±	4.96	26.88	±	2.17	31.28	±	2.74	38.18	±	5.72
9	32.80^{a}	±	5.54	44.10^{a}	±	9.21	64.98^{b}	±	5.46	29.62^{a}	±	2.85
12	32.09	±	4.31	35.46	±	2.76	35.93	±	5.29	42.57	±	3.08
24	30.10	±	6.68	35.27	±	5.07	22.52	±	3.76	34.19	±	6.15
48	22.94a	±	4.25	25.66^{a}	±	4.35	24.90^{a}	±	4.53	43.81^{b}	±	6.97
ANOVA Pur. P values	Treatment 0.0851		Time 0.0065	Treatment X Time <.0001								

Means (± standard error mean, s.e.m) within rows with different superscript letters differ (P ≤ 0.05).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test

The decrease in purine derivates at the end of the incubation after a peak during the first phase of digestion was observed in this study. This can be partly attributed to an increased microbial lysis as a consequence of substrate exhaustion after 48 hours of incubation (Van Nevel & Demeyer, 1977). Russell *et al.* (2009) revealed that the lysis of bacteria such as *Fibrobacter succinogenes* occurs *in vitro* once the stationary phase is reached. This lysis can be triggered by either the depletion of nitrogen and energy sources or some other factor that limits growth of microbes. The observed variation in MPS responses with EFE and yeast

preparation can not only be explained by the microbial lysis. Makkar & Becker (1999) studied the recovery of purine derivates from lyophilized rumen microbial and *Escherichia coli* preparations added to matrices such as cellulose, starch and neutral-detergent fibre. They found that the presence of undigested feed produces errors in the determination of purine derivates. The recovery of purine derivates was poor (approximately 50%) and results were therefore variable. Based on their results, changes in hydrolysis conditions have been proposed for accurate determination of purine bases using spectrophotometric methods. These adjustments were to use mild hydrolysis conditions (0.6 or 2 M $HClO_4$ at 90-95º C for 1 hour) in order to eliminate the interference due to the presence of feed matrices along with microbes and to maximize a complete hydrolysis of nucleic acids.

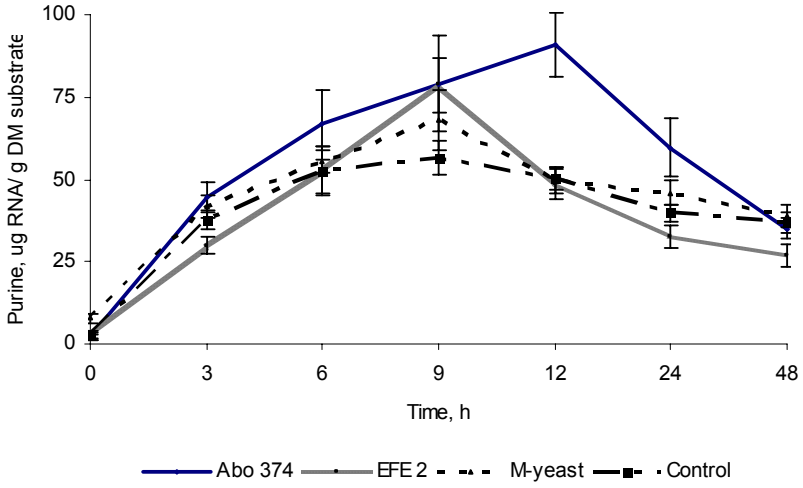

Figure 5.19 Microbial protein synthesis measured as purine derivates on lucerne hay incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

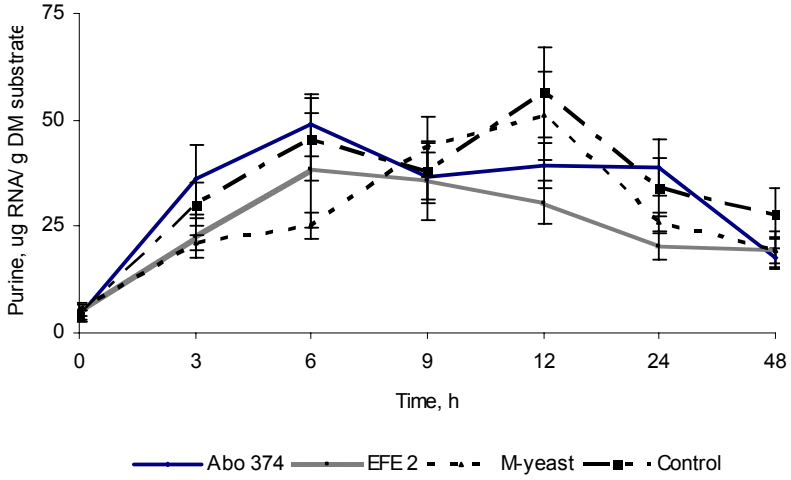

Figure 5.20 Microbial protein synthesis measured as purine derivates on wheat straw incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

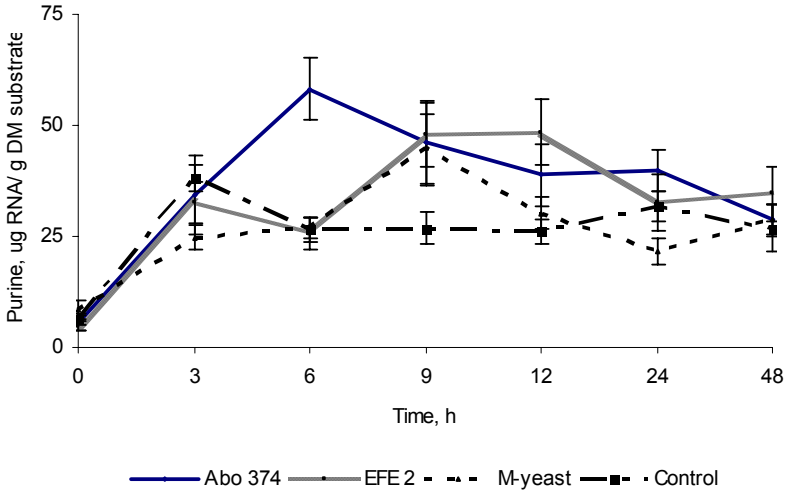

Figure 5.21 Microbial protein synthesis measured as purine derivates on wheat straw treated with urea incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

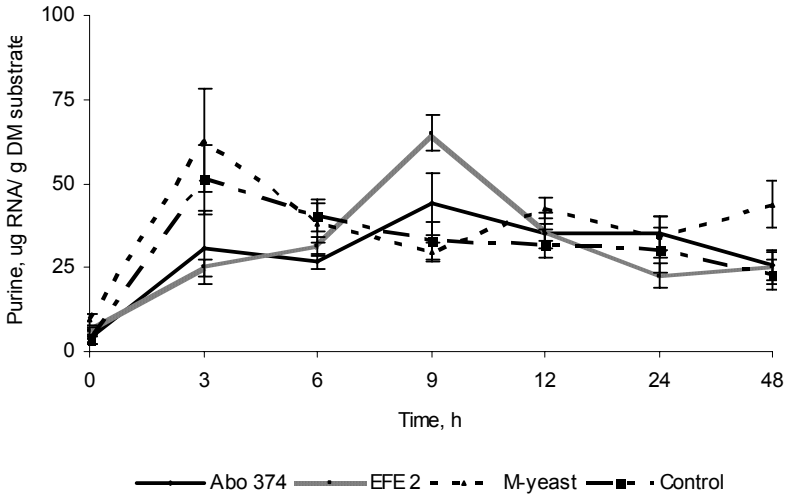

Figure 5.22 Microbial protein synthesis measured as derivates content on concentrate diet incubated with buffered rumen fluid and EFE (Abo 374 or EFE 2) or microbial yeast preparation in an *in vitro* ANKOM digestion for 48 hours. Error bars indicate the standard error of means (s.e.m).

Conclusion

Due to the limitations associated with evaluations of EFE as biotechnological feed additives in *in vivo* trials, the need for a reliable *in vitro* evaluation method is necessary to simulate rumen conditions using rumen fluid. This can be helpful in order to identify products which may have a positive effect on animal performance *in vivo*. The *in vitro* techniques (*in vitro* GP system and filter bag technique) are convenient to use as first

approximations and they are particularly useful for comparative purposes. These *in vitro* methods can be consistently utilized as initial screening method in order to evaluate and identify EFE additives capable to produce a significant effect with regard to feed digestibility using organic matter digestibility (*in vitro* true digestibility) or fermentation characteristics (*in vitro* GP system).

Abo 374 significantly increased the NDF disappearance of lucerne hay in the *in vitro* filter bag procedure whereas the NDF disappearance of wheat straw, wheat straw treated with urea and concentrate diet were not affected by EFE or microbial yeast treatments. The effects of EFE and microbial yeast increased *in vitro* DM disappearance from the *in vitro* filter bag technique of all four substrates at 48 hours ($P < 0.05$), with Abo 374 being the best treatment. Abo 374 and yeast treatment had an effect on CP disappearance of wheat straw and concentrate diet. EFE also significantly increased the cumulative GP, but no correlation between the GP and MPS as purine derivates was observed ($P < 0.05$; $R^2 < 0.30$). The MPS of all four tested substrates were significantly improved in the first half-period of incubation with EFE effects using the *in vitro* filter bag procedure ($P < 0.05$). With the GP system, Abo 374 significantly increased MPS on the concentrate diet determined on residues of GP ($P < 0.0001$) whereas the EFE treatments did not affect MPS of lucerne hay, wheat straw and wheat straw treated with urea. The observed MPS responses can be attributed to the microbial lysis with long periods of incubation and the poor recovery of purine derivates with the Zinn & Owen (1986) analysis procedures.

Results obtained in the present study revealed that EFE can affect the degradability of CP and the output of MPS in addition to enhanced DM and NDF disappearance and increased GP profiles. Direct hydrolysis of fibre fractions due to EFE addition during the pre-treatment period may not be the only explanation by which the GP, purine derivates (MPS) and the *in vitro* disappearance of DM, CP and NDF were enhanced. The addition of EFE may have initiated the erosive alterations of the network of plant cell walls, thereby making it more amendable to microbial degradation. In addition, the effect of EFE may also have increased the hydrolytic capacity within the rumen environment to subsequently enhance the digestion processes. It could be stipulated that the improvement of GP and feed digestion were obtained through a combined effect of direct enzyme hydrolysis and synergetic effect between exogenous (applied) and endogenous (rumen) fibrolytic enzymes. Hence, there is a rising body of evidence demonstrating that the extent of the improvement in feed digestion with EFE implies their viable future in ruminant systems. With a complete understanding of the mechanism of action of these biotechnological products, this would allow the development of EFE products designed particularly to enhance ruminal digestion of low quality forages and harvest crop residues.

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CHAPTER 6

Effect of an exogenous fibrolytic enzyme (Abo 374) on *in vitro* and *in situ* digestion of protein and fibre in ruminant animals

Abstract

The degradation of plant cell walls by ruminants is of major economic importance worldwide as forage is the major source of nutrition in many circumstances. Rumen fermentation is unique in that the efficient fibre degradation relies on the cooperation between micro-organisms that produce fibrolytic enzymes and the host animal that provides anaerobic fermentation conditions. Increasing the efficiency with which the ruminal microbes degrades fibre has been the subject of extensive research for over a century. However the digestibility of plant cell walls continues to limit the intake of digestible energy in ruminants because not even 50% of this fraction is readily digested and utilized. The purpose of this study was to improve fibre digestion using exogenous fibrolytic enzymes (EFE). Therefore an EFE (Abo 374) was evaluated for its impact on microbial protein synthesis (MPS) and disappearances of DM, NDF and CP in the rumen. Abo 374 was tested in a 1:1 mixed substrate of lucerne hay and wheat straw hay using the *in vitro* GP system and ANKOM digestion and the *in situ* technique. The *in vitro* and *in situ* digestion trials were conducted in parallel. A buffered media solution prepared at the same time was used for the *in vitro* GP system and ANKOM digestion. Rumen liquor acquired for the *in vitro* incubations was collected separately as per treatment from four cannulated Döhne-Merino sheep maintained on a standard diet.

Abo 374 significantly improved the cumulative GP but it did not significantly improve the MPS measured as purine derivates of the GP residues ($P < 0.05$). Measured at 48 hours, no correlation was found between MPS and the cumulative GP ($P = 0.68$; $R^2 < 0.25$). Abo 374 enzyme increased the *in vitro* DM and NDF disappearances at 36 hours ($P < 0.05$). *In situ* disappearances of DM, NDF and CP with Abo 374 were similar to the control. This may be due to the small number of sheep used in the study and the relatively high coefficients of variation associated with measuring ruminal digestion. The *in situ* MPS was significantly increased with Abo 374 ($P = 0.0088$). The improved feed digestion, as evidenced by the high disappearance of DM and NDF associated with the increased MPS, resulted from Abo 374 activity during either pre-treatment or digestion process. The net effect of this EFE may be efficient to produce some beneficial erosive depolymerisation of the surface structure of the plant material and the hydrolytic capacity of the rumen. The addition of Abo 374 was likely found to improve the feed digestion as a result of the increased microbial attachment, stimulation of rumen microbial populations and synergistic effects with hydrolases of ruminal micro-organisms. Abo 374 has therefore shown the potential as enzymatic feed additive to enhance fibre degradability of low quality forages fed to ruminants. With reference to these observations, the inclusion of Abo 374 to low quality mixed forage can improve the ruminal digestion of DM, NDF and CP to subsequently enhance MPS.

Key words: crude protein (CP), exogenous fibrolytic enzymes (EFE), dry matter (DM), gas production (GP), neutral-detergent fibre (NDF), microbial protein synthesis (MPS).

Introduction

Ruminant production systems are based on forages as main source of nourishment (Wilkins, 2000). The role of forage is underlined when considerations, resulting from a lack of fibrous material, are given to conditions such as rumen acidosis, parakeratosis and abscesses of liver (McDonald *et al*., 2002). However these feedstuffs contain high fibre associated with low nitrogen (N) and limited available energy (Romney & Gill, 2000). In addition, the quality and yield of forages from pastures vary due to seasonal changes throughout the year. During the dry season, available natural pastures and harvest crop residues are of poor nutritive value as they consist of highly lignified stems (Meissner, 1997). This can significantly affect livestock performance in production systems that utilize forages as a major source of nutrients of the diet.

Fibre or plant cell wall is important for salivation, rumen buffering and efficient production of rumen end products (Mertens, 1997), but less than 65% of plant cell walls are efficiently digested in the total digestive tract (Van Soest, 1994). With 40 to 70% cell walls contained in forage dry matter, attempts to improve ruminal fibre digestion have been an on-going research area. Forage digestibility has been improved by several biotechnological products: ionophores, direct fed microbial products and enzymes (McDonald *et al*., 2002). In the past 10 to 15 years, the use of exogenous fibrolytic enzymes (EFE) as feed additives has shown promise at hydrolyzing plant cell walls (Beauchemin *et al*., 2003). However, the effectiveness of EFE products is highly variable (Giraldo *et al*., 2008a, b). Furthermore, the relationship between improvement in forage utilization and enzymatic activities is yet to be explained with EFE (Eun *et al*., 2007). Several studies with EFE have reported improvements of feed utilization, milk yield and body weight gain in ruminant systems (Lewis *et al*., 1999; Rode *et al*., 1999; Yang *et al*., 1999; Cruywagen & Goosen 2004; Balci *et al*., 2007; Bala *et al*., 2009). Others reported either negative effects or none at all (Vicini *et al*., 2003; Bowman *et al*., 2003; Baloyi, 2008; Eun *et al*., 2008).

Even small improvements in rumen fermentation can influence the feed digestibility (Dawson & Tricarico, 1999). This may improve animal performance as a result of the enhancement of the efficiency at which forage cell walls are digested. In an attempt to improve the nutritive value of ruminant feedstuffs, an EFE (Abo 374) cultivated on wheat straw was evaluated on 1:1 mixed substrate of lucerne hay and wheat straw using *in vitro* and *in situ* techniques.

Materials and methods

According to the protocol of the animal care and use committee of Stellenbosch University (SU ACUC, Ethic clearance number: 2006B03005), four cannulated Döhne-Merino sheep were randomly assigned in two groups in a 2 x 2 cross-over experiment. Animals were used to evaluate the effects of EFE (Abo 374) *in vitro* and *in situ* using a 1:1 mixed substrate of lucerne hay and wheat straw. Abo 374 is a South African fungal EFE cultivated on wheat straw and developed at the Department of Microbiology (Stellenbosch University). Sheep received a daily basal diet (Table 6.1) supplemented with 300 g/day of concentrate [880 g/kg of DM,

100 g/kg of CP, 250 g/kg of NDF, 15 g/kg Ca and 2 g/kg P]. The basal diet and water were offered *ad libitum*. The concentrate diet was given in the morning. The standard diet was treated at a daily level of 5 ml/kg feed to provide either no enzyme (Control: distilled water) or Abo 374 enzyme concentrate. This was pre-treated the evening before feeding to allow an enzyme interaction time with the substrate (Beauchemin *et al*., 2003). To ensure good homogeneity, 5 ml/kg enzyme concentrate or distilled water was then added to 100 ml of distilled water before being sprayed on the diet. The experiment was conducted following ten days of an adaptation to the basal diet (Table 6.1).

Table 6.1 Composition of basal experimental diet fed to sheep.

Components	Amount (%)
Physical composition	
Lucerne hay	29.85
Wheat straw	29.85
Corn starch	14.92
Molasses meal	23.88
Premix	1.5
Chemical composition (DM basis)	
DM (g/kg)	830.31
OM (g /kg)	913.87
Ash (g /kg)	86.13
CP (g/kg)	75.51
NDF (g/kg)	361.54

Treatments of the standard diet began two days before the first replication of the *in sacco* incubation. This was to allow the beginning of the pre-consumption effects of EFE and improve synergy between EFE and ruminal enzymes. After the first incubation, animals were randomized and a three days re-adaptation period was used in two phases of one day and two days. During the first day, an enzyme free standard diet was fed to eliminate any EFE in the digestive tract prior to the second run of the *in sacco* incubation. This was followed by two days of EFE or distilled water treated standard diet. Treatments of feed substrate with Abo 374 or distilled water were done 12 hours before incubation. This was to create a stable and interactive enzyme-feed complex and to weaken fibre structures which would possibly stimulate microbial colonization (Beauchemin *et al*., 2003). For the *in vitro* procedures, a ratio of one ml of the Abo 374 dilution or distilled water to 0.5 g substrate was used. The GP system and ANKOM technique were simultaneously conducted for 48 hours according to methodology descried in Chapter three, with rumen liquor collected at 06h00 separately as per treatment. The 50 x 55 mm ANKOM® Dacron bags (ANKOM® F57 filter bag, ANKOM® Technology Corp., Fairport, NY, USA) were used for the ANKOM digestion. These are made from multi layer polyethylene polyester in a filamentous matrix which can retain particles less than 30 µm (ANKOM Technology Corporation, 1997). The *in situ* nylon bags were treated with 1ml of enzyme dilution or distilled water per g of substrate.

The *in situ* or *in sacco* technique consists of suspending animal feedstuffs inside Dacron bags for different periods of time in the rumen. This implies that the feed sample is in contact with the rumen environment and therefore can be fermented and degraded by rumen micro-organisms in the bags as it would be in the rumen. The 10 x 20 cm ANKOM® Dacron bags (ANKOM® Technology Corp., Fairport, NY, USA) were used in the *in sacco* procedure according to Vanzant *et al* (1998). These bags are made of nitrogen-free polyester and have a pore size of 50 ± 15 µm. Bags were marked and placed in the oven at 100º C overnight. Once dried and weighed with a marble inside the bag, they were filled with 8 ± 0.05 g of mixed substrate. The bags were sealed by double folding the top two cm of the bag and then knotted using a fishing twine. The twine was tied around the bag and attached to a circular metal weight so as to separate bags so that one bag could be removed at a time. The reason for placing a marble inside the bag and tying bags to the weight was to keep them as submerged as possible in the rumen contents. Seven substrate filled bags and one blank were suspended into the rumen simultaneously. The blank correction bag was to account for weight changes due to microbial contamination occurring during the incubation. Bags were removed after pre-determined incubation times (6, 9, 12, 18, 24, 36 and 48 hours). A zero hour bag was not incubated in the rumen, but washed with running water and kept frozen. During removal of a bag, care was taken to not retrieve and expose the other bags remaining in the rumen to air. As bags were withdrawn from the rumen, they were washed with running water and kept frozen for further processing and analysis. After the trial, all bags were defrosted at the room temperature and later simultaneously machine washed with cold water until the washing water was clear. This was to improve the standardization of the washing procedure. The bags were spun to remove excess washing water before being placed in a drying oven at 60º C for three days. Once dried, the bags were placed in the desiccator for 30 minutes before being weighed. The bag residues were then analyzed for DM, CP, NDF and purine derivates according to chemical analyses described in Chapter three. All data generated from the digestibility studies was subjected to the two-way repeated measures of the analysis of variance (ANOVA) and the instantaneous rate of degradation was obtained using a non linear model of the SAS enterprise guide 4 (2006, SAS Institute Inc.), as described in Chapter five.

Results and discussion

The effect of Abo 374 treatment of the mixed substrate of lucerne hay and wheat straw on *in vitro* GP is presented in Table 6.2 and Figure 6.1. Enzyme treatment increased the cumulative GP of the mixed substrate of lucerne hay and wheat straw at 48 hours. Eun & Beauchemin (2007) reported that EFE are mostly to be effective during the first 6 to 12 hours of digestion. Colombatto *et al*. (2003) elucidated that EFE do not affect final GP or the extent of fibre digestion after a long period (96 hours) of incubation. In addition, EFE were found to be resistant to rumen proteolysis and therefore actively stable to continue to hydrolyse feed in the rumen fluid (Morgavi *et al*. 2000a, b). This evidence supports the substantial synergism between EFE and ruminal enzymes at which the net combined hydrolytic activity is increased in the rumen (Beauchemin *et al*., 2004). Giraldo *et al*. (2007a, b) reported that treating a high-forage substrate with EFE from *T. longibrachiatum* increased the MPS measured as $^{15}N\text{-}NH_3$ into substrate after 6 hours of incubation in Rusitec fermenters and improved fibre degradation. These authors concluded that EFE stimulated the initial phase of microbial colonization. Consistent with this, Giraldo *et al*., (2008a) reported that a positive synergy

between EFE and rumen enzymes were responsible for the increased *in vitro* GP, total VFA, true degradability of substrate DM and decreased methane production. This stipulated that EFE subtly erode cell wall structure allowing ruminal microbes to obtain earlier access to fermentable substrate during the initial phase of digestion (Colombatto *et al*., 2003). In agreement with these studies, Abo 374 significantly increased the GP of mixed substrate of lucerne hay and wheat straw after 12 hours of incubation (P = 0.0014) but not in the first 6 to 12 hours. The GP profiles were significantly different with the effects of treatment (P =0.0014) and incubation time (P < 0.0001) as well as their interaction (P = 0.0201). Differences of over 10% between Abo 374 and control (P < 0.05) were permanently recorded from 12 to 48 hours of incubation (Table 6.2 and Figure 6.1). At 12 hours, the rate of gas produced with Abo 374 was 5.73 compared to 5.19 per hour in control. The rate of gas produced from both Abo 374 and no enzyme were decreased with time (48 hours) respectively to 0.05 and 1.44 per hour.

Table 6.2 Effects of Abo 374 on cumulative GP, CP disappearance, NDF disappearance and MPS of GP residues of mixed substrate of lucerne hay and wheat straw.

Cumulative GP, ml/g OM						
Time, hours	Control treatment			Abo treatment		
1.5	15.63	±	0.54	18.28	±	1.62
3	30.3	±	0.86	32.02	±	2.33
6	54.45	±	1.08	60.04	±	4.78
9	69.53	±	1.2	76.03	±	4.86
12	83.60[a]	±	1.09	95.69[b]	±	6.01
18	106.86[a]	±	1.22	121.00[b]	±	5.65
24	126.73[a]	±	1.39	140.56[b]	±	6.04
36	160.67[a]	±	2.44	182.83[b]	±	8.21
48	173.91[a]	±	3.72	200.24[b]	±	8.92
ANOVA GP P values	Treatment 0.0014		Time <.0001	Treat X Time 0.0201		
CP degradation, NDF digestibility and purine content of GP residues at 48 hours						
	Control treatment			Abo treatment		P-values
CP,%	36.93	±	0.11	35.31	±	0.74	0.163
NDF,%	52.3	±	1.11	54.5	±	1.36	0.3381
Purine, µg/DM g	210.65	±	13.7	218.43	±	12.19	0.68

Means (± standard error mean, s.e.m) within rows with different superscript letters differ (P ≤ 0.05).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test.

Microbial fermentation of organic matter (OM) produces fatty acids (VFA; acetic, propionic, butyric acid), microbial protein synthesis (MPS), carbon dioxide (CO_2) methane (CH_4) and small amount of hydrogen (H_2) in the rumen (Van Soest, 1994). Krishnamoorthy *et al*. (1991) reported a positive linear relationship between MPS and cumulative GP (up to 8 hours of incubation) using mixed carbohydrate as substrate without EFE addition. As microbial biomass is increased with EFE addition, this can have a significant influence on the fermentation efficiency (Eun & Beauchemin, 2007). Consistent with this hypothesis, Giraldo *et al*. (2007a, b) observed that EFE from *T. longibrachiatum* of high forage substrate increased the *in vitro* production of VFA, the fibrolytic activity of the rumen fluid and number of cellulolytic microbes. In another study, treating the diet of dairy cows with EFE from *T. longibrachiatum* increased the numbers of rumen bacteria utilizing hemicellulose or secondary products of cellulose (Nsereko *et al*., 2002). Results of this study showed that

Abo 374 increased the MPS measured as purine derivates by 3.69% (Figure 6.2). However the effect was not significant (P = 0.68). Measured at 48 hours, no correlation was found between MPS and the cumulative GP (P = 0.68; R^2 < 0.25). Neither CP degradation nor NDF digestibility from the GP system were significantly affected at 48 hours. Although microbial lysis can increase as a consequence of substrate exhaustion with a long incubation trial (Van Nevel & Demeyer, 1977), the lack of significance of MPS may also be related to the low recovery of purine derivates with the Zinn & Owens analysis (1986). Makkar & Becker (1999) found that low recovery of purine derivates can be observed as the presence of undigested feed produces analytical errors in the determination of purine.

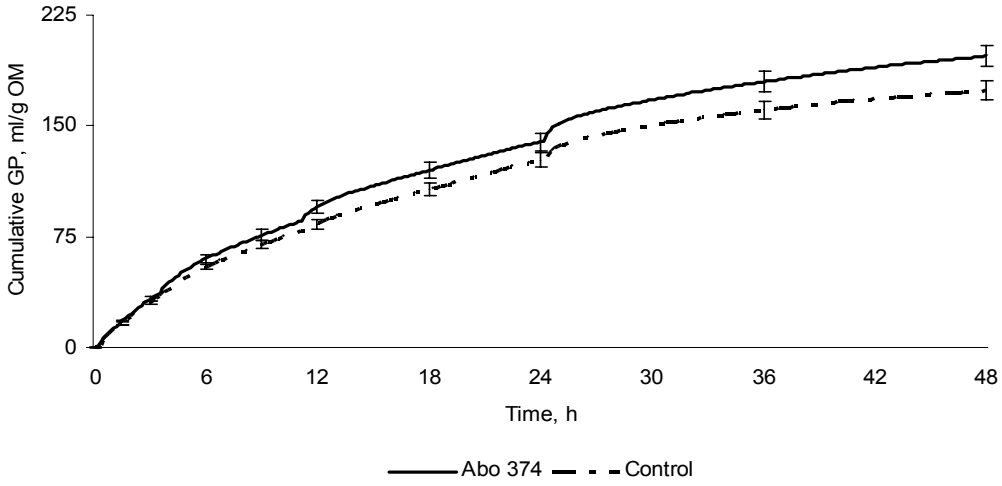

Figure 6.1 Cumulative GP* (ml/g OM) of the mixed substrate of lucerne hay and wheat straw incubated with buffered rumen fluid and EFE (Abo 374) for 48 hours. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: Cum. GP (Abo 374) = 9.12 + 7.63 time – 0.079 time^2, R^2= 0.96 and Cum. GP (control) = 7.97 + 6.94 time – 0.073 time^2, R^2= 0.99.

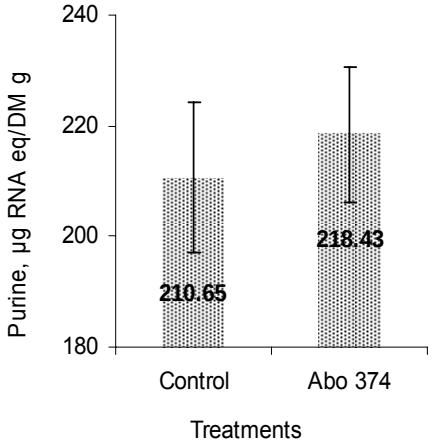

Figure 6.2 Microbial protein synthesis measured as purine derivates (RNA equivalent in µg/DM g) on residues of GP of the mixed substrate of lucerne hay and wheat straw. Substrate was incubated with buffered rumen fluid and EFE (Abo 374) or no enzyme for 48 hours. Error bars indicate the standard error of means (s.e.m).

The effects of Abo 374 treatment of the mixed substrate of lucerne hay and wheat straw on the *in vitro* disappearance (DM, CP and NDF) and MPS measured as purine derivates are presented in Table 6.3 and Figures 6.3-4. The treatment effect was not significant on the *in vitro* disappearance (DM, CP and NDF) or MPS. The effect of incubation time was found to significantly increase the *in vitro* disappearance ($P < 0.0001$) as expected. No significant interaction effects of treatment and time were observed on the undigested residues for either DM or NDF. The interaction of treatment and time were significantly on the *in vitro* CP disappearance ($P = 0.0074$). At 36 hours (Figure 6.3), both DM and NDF disappearances were significantly improved with the enzyme treatment. Abo 374 increased the disappearance of DM by 6.28% ($P = 0.0321$, Bonferroni t-test) at a degradation rate of 0.314 compared to 0.274 per hour for the control. The DM disappearances for Abo 374 and control were respectively 180 and 144.84 times higher than their respective disappearances at zero hour. For NDF disappearance at 36 hours, Abo 374 increased the disappearance by 2.85% ($P = 0.0495$, Bonferroni t-test) at a degradation rate of 0.484 compared to 0.490 per hour for the control. The NDF disappearances for Abo 374 and control were respectively 213.50 and 145.14 times higher than their respective disappearance at zero hour. Similar to this finding, Goosen (2005) reported a positive effect with Abo 374 enzyme on the *in vitro* DM and NDF degradation of wheat straw.

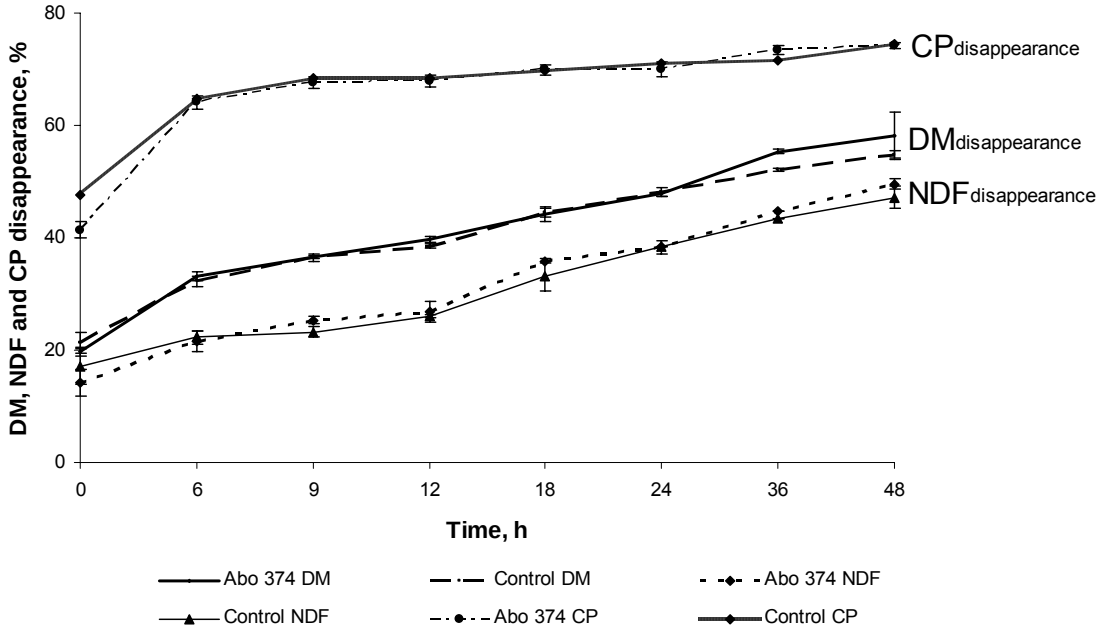

Figure 6.3 Dry matter, NDF and CP *in vitro* disappearances* of the mixed substrate of lucerne hay and wheat straw. Substrate was incubated with buffered rumen fluid and EFE (Abo 374) for 48 hours (*in vitro* filter bag technique). Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: DM (Abo 374) = 23 + 1.5 time – 0.018 time^2, $R^2 = 0.95$; DM (control) = 22.41 + 1.54 time – 0.01 time^2, $R^2 = 0.90$; NDF (Abo 374) = 15.89 + 1.089 time – 0.009 time^2, $R^2 = 0.96$; NDF (control) = 14.29 + 1.301 time – 0.012 time^2, $R^2 = 0.97$; CP (Abo 374) = 49.24 + 1.64 time – 0.024 time^2, $R^2 = 0.74$ and CP (control) = 49.24 + 1.64 time – 0.024 time^2, $R^2 = 0.75$.

Table 6.3 Effects of Abo 374 on *in vitro* MPS measured as purine derivates and disappearance (DM, CP and NDF) of the mixed substrate of lucerne hay and wheat straw (*in vitro* filter bag technique).

In vitro DM disappearance						
Time, hours	Control DM, %			Abo 374 DM, %		
0	21.28	±	1.77	19.78	±	0.82
6	32.34	±	0.91	33.15	±	0.89
9	36.53	±	0.26	36.49	±	0.6
12	38.54	±	0.5	39.65	±	0.49
18	44.46	±	0.79	44.11	±	1.31
24	48.23	±	0.77	47.8	±	0.32
36	52.1[a]	±	0.38	55.38[b]	±	0.4
48	54.79	±	0.64	58.16	±	4.15
ANOVA DM	Treatment		Time	Treatment X Time		
P values	0.3005		<0.0001	0.5423		
In vitro NDF disappearance						
Time, hours	Control NDF, %			Abo 374 NDF, %		
0	17.12	±	3.13	14.27	±	2.43
6	22.33	±	1.22	21.61	±	1.77
9	23.27	±	0.93	25.33	±	0.71
12	26.09	±	0.32	26.85	±	1.85
18	33.21	±	2.65	35.83	±	0.37
24	38.41	±	0.1	38.34	±	1.16
36	43.5[a]	±	0.05	44.74[b]	±	0.01
48	47.17	±	1.93	49.6	±	0.83
ANOVA NDF	Treatment		Time	Treatment X Time		
P values	0.5478		<0.0001	0.6906		
In vitro CP disappearance						
Time, hours	Control CP, %			Abo 374 CP, %		
0	47.72[a]	±	1.4	41.38[b]	±	1.4
6	64.67	±	1.33	64.14	±	1.19
9	68.47	±	0.66	67.57	±	1.05
12	68.35	±	1.14	67.96	±	1.03
18	69.78	±	0.42	69.96	±	0.92
24	71.14	±	0.26	70.00	±	1.21
36	71.52	±	0.41	73.45	±	0.81
48	74.48	±	0.73	74.19	±	0.53
ANOVA CP	Treatment		Time	Treatment X Time		
P values	0.1165		<0.0001	0.0074		
Purine derivates on residues after ANKOM digestion						
Time, hours	Control purine, µg RNA equivalent/DM g			Abo 374 purine, µg RNA equivalent /DM g		
6	92.14[a]	±	20.19[b]	62.43	±	12.62
9	57.11	±	10.59	45.13	±	12.08
12	88.46[a]	±	10.49[b]	46.41	±	13.56
18	70.27	±	10.25	73.37	±	14.62
24	64.04	±	9.33	58.28	±	10.75
36	66.95	±	9.77	68.86	±	10.35
48	65.53	±	9.53	73.31	±	22.05
ANOVA Pur.	Treatment		Time	Treatment X Time		
P values	0.1241		<0.0001	0.1268		

Means (± standard error mean, s.e.m) within rows with different superscript letters differ (P ≤ 0.05).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test.

The disappearance of CP was significantly 13.28% lower with Abo 374 than the control treatment at zero hour of incubation (P < 0.0001, Bonferroni t-test) (Figure 6.3 and Table 6.2). The reason for this negative

effect was unclear. Negative effects on MPS measured as purine derivates were also observed at 6 and 12 hours with Abo 374 treatment ($P < 0.05$). The MPS were significantly lower with Abo 374 compared to control (Figure 6.4). This was probably due to high variations observed during the procedure for purine analysis. In another study, the low recovery of purine derivates was observed because the presence of undigested feed interferes in the determination of purine derivates (Makkar & Becker, 1999). Hence, the presence of undigested feed could have been a contributing factor in the variation of MPS observed in this study.

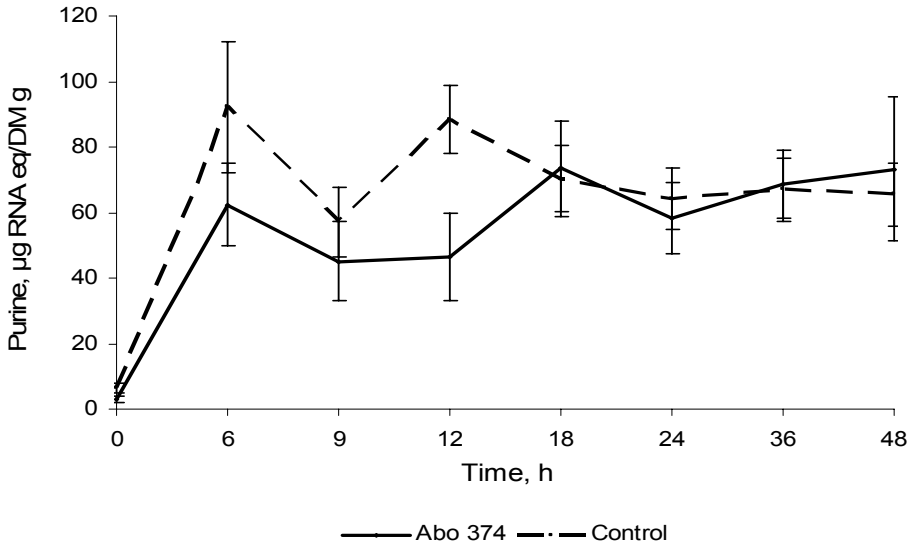

Figure 6.4 Microbial protein synthesis measured as purine derivates (RNA equivalent in µg/DM g) on residues of *in vitro* nylon bag digestion of the mixed substrate of lucerne hay and wheat straw. Substrate was incubated with buffered rumen fluid and EFE (Abo 374) or no enzyme for 48 hours (*In vitro* filter bag technique). Error bars indicate the standard error of means (s.e.m).

In vitro results revealed in both the GP system and the ANKOM technique that Abo 374 was not efficient to improve DM and NDF disappearance in the first 6 to 12 hours of digestion, in contrast to other studies (Dawson & Tricarico, 1999; Collombatto *et al*., 2003; Nowak *et al*., 2003; Beauchemin *et al*., 2003). In general, the significant effect of Abo 374 on the GP system and *in vitro* filter bag procedure was observed in this study in between 12 hours and the end of the incubation (48 hours). In agreement with this finding, Lewis *et al*. (1996) found a lack of EFE effects on the *in situ* disappearance of DM and NDF during the initial phase of digestion. These authors found that the positive effects were observed after 32, 40 and 96 hours of incubation. In another study, high DM disappearance was also found in an EFE treated grass substrate incubated in the rumen only after 24 and 48 hours (Feng *et al*., 1996). They stipulated that the increase after a long period of incubation could result from enhanced colonization and digestion of slowly degradable plant cell wall fraction by ruminal micro-organisms.

Another factor which contributes to improvement of DM and NDF digestion in ruminant systems is the mode of application of EFE to feeds. In this study, a liquid of Abo 374 dilution was sprayed directly onto a dry mixed substrate of lucerne hay and wheat straw 12 hours before feeding to allow enzyme-feed interaction.

Beauchemin *et al*. (1999) reported that the addition of EFE to dry feedstuffs before feeding enhances the binding of EFE with the substrate, which can improve the resistance of EFE to proteolysis and prolong their residence in the rumen (Morgavi *et al*,. 2001; Beauchemin *et al*., 2003). In another study, high digestibility observed with lactating dairy cows fed with an EFE treated diet was found to be resultant of EFE effect via diverse mechanisms (Kung *et al*., 2000). These may include the direct hydrolysis, improved microbial adhesion, synergistic action with ruminal enzymes and changes in the site of nutrient digestion (Beauchemin *et al*., 2003). The *in vitro* results indicated that Abo 374 was partly resistant to proteolysis in the incubation milieu and remained active after a relatively long period (36 hours).

The effects of Abo 374 on the *in situ* disappearance (DM, CP and NDF) and MPS measured as purine content are presented in Table 6.4 and Figures 6.5-6. No different effects of treatment were observed on DM, NDF and CP of the *in situ* undigested residues of the mixed substrate of lucerne hay and wheat straw whereas there was significant period effect ($P < 0.0001$). In contrast to the *in vitro* results, there was a significant effect of treatment ($P = 0.0088$) and time ($P < 0.0001$) on MPS. No interaction on rumen undigested residues from treatment and incubation time was observed on MPS. Sets of data from the parallel *in vitro* and *in situ* techniques were compared for disappearances of nutrient fractions (DM, CP and NDF). The comparison of results showed an overestimation by the *in situ* method. With regard to this finding, Trujillo *et al*. (2010) revealed that the disappearance can be overestimated despite the existence of a good correlation ($R^2 = 0.94$) between the *in vitro* and *in situ* methods (Spanghero *et al*., 2003). In the *in situ* procedure, larger pore size bags and physical rumen contractions during digestion can allow faster rates of rumen liquor flow through the bags. This could result in larger losses of particles and degraded compounds from the bags. As the *in sacco* bags have 50 ± 15 µm pore size compared to 30 µm for the F57 bags (ANKOM Technology Corporation, 1997), this may explain the higher DM disappearance of substrate observed *in situ* at zero hour. Furthermore the microbial ability to degrade substrates may be affected by multiple factors which could destabilize or unsettle the microbial inoculum in the *in vitro* procedure and therefore bias the *in vitro* data, particularly in the initial phase of digestion (Wallace *et al*., 2001). These factors include the source of rumen inoculum, composition and nutrient availability of diets offered to donor animal, rumen sampling time, inoculum preparation, sustained anaerobic environmental conditions during inoculum preparation, composition of the buffer solution, relative proportions of inoculum and medium and the pH during incubation (Trujillo *et al*., 2010).

Table 6.4 Effects of Abo 374 on *in situ* MPS measured as purine derivates and disappearance (DM, CP and NDF) of the mixed substrate of lucerne hay and wheat straw.

In situ DM disappearance						
Time, hours	Control DM, %			Abo 374 DM, %		
0	35.20	±	0.21	35.26	±	0.19
6	43.22	±	0.44	43.38	±	1.25
9	45.01	±	0.51	45.51	±	0.81
12	47.06	±	0.69	47.83	±	1.25
18	49.40	±	0.41	49.93	±	1.90
24	52.11	±	1.18	52.79	±	1.69
36	55.91	±	2.07	56.27	±	1.13
48	60.10	±	1.02	60.35	±	1.07
ANOVA DM	Treatment		Time	Treatment X Time		
P values	0.44		<0.0001	0.9999		
In situ NDF disappearance						
Time, hours	Control NDF, %			Abo 374 NDF, %		
0	18.56	±	0.42	18.44	±	0.51
6	22.29	±	0.55	21.86	±	0.86
9	22.76	±	0.72	24.36	±	0.86
12	25.91	±	0.57	26.13	±	0.94
18	28.94	±	0.50	28.91	±	1.94
24	32.26	±	1.26	33.65	±	1.64
36	38.27	±	1.99	38.20	±	1.18
48	43.78	±	1.11	44.65	±	1.07
ANOVA NDF	Treatment		Time	Treatment X Time		
P values	0.3818		<0.0001	0.9322		
In situ CP disappearance						
Time, hours	Control CP, %			Abo 374 CP, %		
0	38.40	±	3.48	37.92	±	2.65
6	50.79	±	1.87	53.21	±	2.02
9	58.05	±	0.48	59.13	±	0.53
12	60.69	±	0.53	61.57	±	0.76
18	60.70	±	0.97	62.25	±	1.07
24	63.20	±	0.44	64.32	±	0.96
36	65.06	±	1.34	66.97	±	0.64
48	67.50	±	1.07	67.10	±	0.79
ANOVA CP	Treatment		Time	Treatment X Time		
P values	0.2103		<0.0001	0.9722		
Purine derivates on residues after *in situ* digestion						
Time, hours	Control purine, µg RNA eq/DM g			Abo 374 purine, µg RNA eq/DM g		
6	32.72	±	2.12	38.60	±	3.61
9	34.92	±	2.61	40.85	±	4.65
12	37.94	±	2.02	37.84	±	4.36
18	47.86	±	4.22	47.24	±	4.84
24	43.39	±	2.88	50.36	±	4.71
36	46.91[a]	±	2.98	55.59[b]	±	3.31
48	49.93[a]	±	3.63	59.90[b]	±	3.88
ANOVA Pur.	Treatment		Time	Treatment X Time		
P values	0.0088		<0.0001	0.3718		

Means (± standard error mean, s.e.m) within rows with different superscript letters differ (P ≤ 0.05).
Differences between treatments were obtained using the pair wise comparison of Bonferroni t-test

Abo 374, which is cultivated on wheat straw substrate, is a fungal enzyme cocktail containing cellulases, xylanases and mannanases, with xylanase as major fibrolytic activity (Cruywagen & Van Zyl., 2008). Although the ruminal differences were not statistically significant (Figure 6.5), the increased *in situ* disappearance of DM and NDF appeared to be caused by the improvement of ruminal activity with Abo 374 addition. Lack of statistical differences in ruminal digestion between Abo 374 enzyme and control may be resulted from the small number of sheep used in the study and the relatively high coefficients of variation associated with measuring ruminal digestion. In two other studies evaluating this enzyme at a similar dose, Abo 374 enzyme was reported to significantly improve body weight gains and feed conversion efficiency when fattening lambs on forage based-diets (Cruywagen & Goosen, 2004; Cruywagen & Van Zyl, 2008). These authors speculated that Abo 374 increased hydrolytic capacity in the rumen, which improved fibre digestibility.

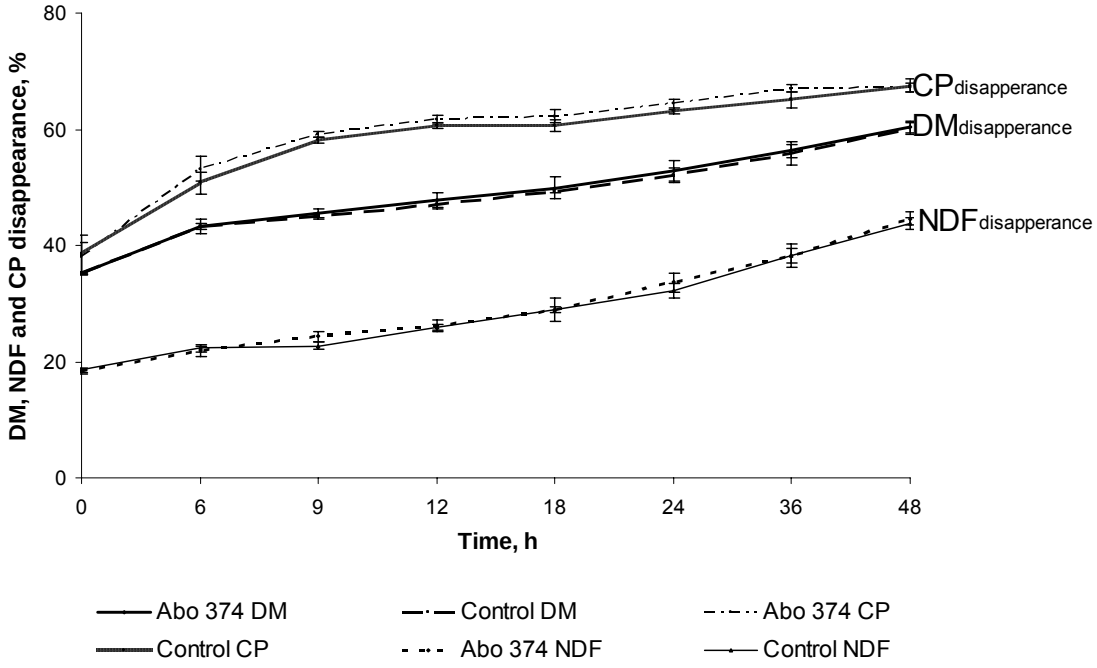

Figure 6.5 Effects of Abo 374 on *in situ* disappearances* of the mixed substrate of lucerne hay and wheat straw. Substrate was incubated for 48 hours in the rumen of sheep fed a standard diet treated with Abo 374. Error bars indicate the standard error of means (s.e.m).

*Quadratic fits: DM (Abo 374) = 37.16 + 0.89 time – 0.008 time^2, R^2 = 0.89; DM (control) = 37.06 + 0.85 time – 0.008 time^2, R^2 = 0.92; NDF (Abo 374) = 18.4 + 0.65 time – 0.0023 time^2, R^2 = 0.72; NDF (control) = 18.37 + 0.62 time – 0.0018 time^2, R^2 = 0.89; CP (Abo 374) = 42.67 + 1.57 time – 0.022 time^2, R^2 = 0.76 and CP (control) =. 42.5 + 1.43 time – 0.019 time^2, R^2 = 0.72.

An increased CP disappearance was observed with Abo 374 during the *in situ* digestion but the differences were not statistically significant (Figure 6.5). The increase of *in situ* CP disappearance seemed to be caused by the improvement of ruminal proteolytic activity with Abo 374 addition. Álvarez *et al.* (2009) found that EFE increased solubility of DM and CP fractions in association to the increased disappearance of NDF and acid-detergent fibre (ADF) fractions. These authors attributed the higher total disappearance of CP to the direct

effect of EFE, which was not only limited to plant cell wall components. This finding explained why EFE might be effective at improving the digestibility of the non structural components of plant cells in relation to the increased digestibility of the fibrous fraction (Beauchemin *et al*., 2003). In another study, EFE enhanced the nitrogen (N) intake, faecal N and N retention of lucerne and ryegrass hays to subsequently increase the apparent digestibility of CP (Pinos-Rodríguez *et al*., 2002). According to McAllister *et al*. (2001), EFE appears to contain some proteolytic activities as they facilitate degradation of cell wall bound proteins. In another investigation, EFE with cellulases as major activity were reported to increase CP degradation of forages *in vitro* by making proteins more available to proteolytic enzymes (Kohn & Allen, 1992). In agreement with these findings, Rode *et al.* (1999) found that EFE did not enhance the DM intake, but increased milk production as a result of an increased digestion of carbohydrates (OM and NDF) and CP.

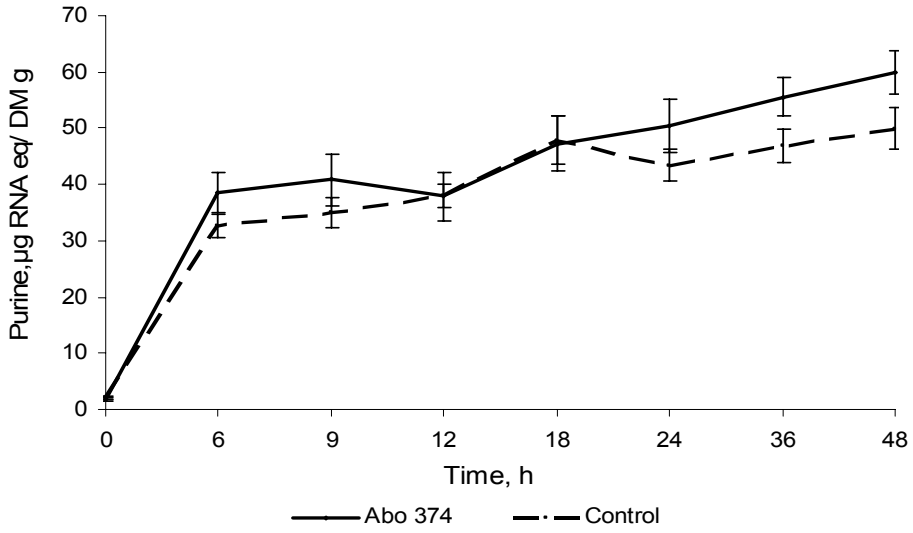

Figure 6.6 Microbial protein synthesis measured as purine derivates (RNA equivalent in µg/g DM) on residues of *in situ* nylon bag digestion of the mixed substrate of lucerne hay and wheat straw. Substrate was incubated for 48 hours in the rumen of sheep fed a standard diet treated with Abo 374. Error bars indicate the standard error of means (s.e.m).

In this study, Abo 374 was found to significantly increase the MPS of the mixed substrate consisting of lucerne hay and wheat straw in the *in situ* experiment (P = 0.0088) (Figure 6.6). Similarly, Yang *et al.* (1999) found that *in situ* ruminal microbial attachment to feed residues was very rapid. More than 2% microbial DM was found present in feed residues after only 30 minutes of incubation in the rumen. The proportion of microbial DM in the total residues, measured as ^{15}N, was reported to increase rapidly during the first 12 hours of incubation and then slowly increased until the last incubation time (72 hours) (Yang *et al.,* 1999). As pointed out by Trujillo *et al*., (2010), the *in situ* results may be affected by the high flow of rumen fluid into the nylon bags and also by a sustained supply of nutrients to the microflora as animals consume feed. The increased microbial colonization on the mixed substrate treated with Abo 374 was likely related to enzyme activity. Enzymes applied to feed can randomly release reducing sugars and possibly make more nutrients available (Hirstov *et al*., 1996), arising from partial solubilisation of cell wall components (Krause *et al*., 1998). Forsberg *et al*. (2000) reported that the presence of soluble sugars would supply sufficient additional

available carbohydrates to shorten the lag time needed for microbial colonization and also enhance the rapid microbial attachment and growth. This may be obtained with the increased production of the glycocalyx, which is produced by bacteria and permits adhesion between bacteria or between bacteria and substrate (Beauchemin *et al*., 2004). Consistent with these reports, Bala *et al*. (2009) observed an increase in milk yield of lactating goats. This occurred as a result of the improvement of the energy availability and the utilization of microbial digestible protein, estimated based on purine derivatives and creatinine excreted in urine (Bala *et al*. 2009). These authors speculated that EFE were able to free the trapped nutrients in the cell wall networks of roughages. This was reported by Chakeredza *et al.* (2002) to improve the yield of fermentative end products which has changed the ratio of microbial protein production to the digestible energy in the rumen.

Because protective barriers (waxy cuticle and husk) and compounds (condensed tannin and phenolic acids) in plants form a major defence against microbial attack (Van Soest, 1994; Selinger *et al*., 1996), rumen microbes may access feed particles through disruption of the protective barrier caused by chewing or mechanical processing (Buxton & Readfearn, 1997). The addition of EFE can weaken plant barriers that limit microbial digestion in the rumen, thereby making feed substrates more amenable to degradation (Beauchemin *et al*., 2004). Nsereko *et al*. (2002) found that EFE indirectly increased the attachment and the number of cellobiose- and glucose- utilizing bacteria in the rumen. Similarly, Giraldo *et al*. (2008b) found that treating high-forage diets with EFE increased fibrolytic activity and stimulated the *in vitro* numbers of micro-organisms. The stimulation of non-fibrolytic and fibrolytic bacteria may therefore increase the availability of substrate as a result of improved cell wall digestion and may accelerate the digestion of newly ingested feedstuffs (Beauchemin *et al*., 2004). This may amplify the synergy between EFE and ruminal enzymes (McAllister *et al*., 2001). Furthermore, the enhanced attachment and total number of microbes by EFE can result in greater micro-organism biomass and therefore would impact the supply of metabolizable protein to the small intestine (Yang *et al*., 1999). The addition of Abo 374 to the mixed substrate of lucerne hay and wheat straw appeared to have been beneficial for microbial colonization of feed particles. The net effect of EFE could have likely initiated the primary microbial colonization and the release of digestion products that attracted in return additional bacteria to the site of digestion.

Conclusion

There is a body of evidence with biotechnological enzyme products indicating that EFE in ruminant diets can increase forage utilization, improve production efficiency and reduce nutrient excretion. Abo 374, which is a South African EFE cultivated on wheat straw, has the potential to enhance fibre degradability of low quality forages fed to ruminants. Evidences such as increased feed digestibility and animal body weight were previously reported with this enzyme *in vitro* and *in situ*.

Results from this study showed that Abo 374 treatment of the mixed substrate of lucerne hay and wheat straw significantly increased the *in vitro* DM and NDF disappearances at 36 hours and the GP profiles ($P < 0.05$). In addition, no correlation was found between MPS and the cumulative GP at 48 hours ($P = 0.68$; $R^2 <$

0.25). The effects of Abo 374 on the *in situ* disappearance (DM, NDF and CP) were similar to control. This may be due to the small number of sheep used in the study and the relatively high coefficient of variation associated with measuring ruminal digestion. Abo 374 significantly increased the *in situ* MPS, measured as purine derivates (P = 0.0088). Evidence of the increased MPS and both *in vitro* and *in situ* disappearance of DM and NDF was likely related to the Abo 374 activity during either pre-treatment or digestion process. These findings revealed that this EFE was efficient to improve the solubility of DM and NDF fractions in association with the degradation of CP to subsequently enhance MPS. The net effect of Abo 374 could have increased the feed digestion as a result of the improvement of direct hydrolysis, microbial attachment and stimulation of the rumen microbial population and synergistic effects with hydrolases of ruminal micro-organisms.

In vitro bioassays that reflect the ruminal conditions are a good alternative to *in vivo* studies to identify ideal EFE candidates for use in ruminant diets. However, positive results from *in vitro* systems (GP and ANKOM digestion) and the *in situ* nylon bag technique must be confirmed in the *in vivo* system for validation. Further studies using a larger number of ruminants fed for a longer duration are needed to confirm the effects of the addition of Abo 374 to forage based diets. As pointed out by Wallace *et al*. (2001), an identification of the key activity and optimum level of EFE for a positive response in rumen ecosystem is of great importance. Although it is still a challenge, this may be the bridge to explain the relationship between improvement in forage utilization and EFE in ruminants. Further studies must also determine whether Abo 374 enzyme is most effective when added to forage, concentrate, or the total mixed diet before this enzyme should be made available to commercial ruminant farmers.

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CHAPTER 7

General conclusion

Ruminant production systems throughout the world are based on available natural pastures and harvest crop residues. These are of poor nutritive value as they consist of highly lignified stems. Forage utilization is limited by low quality (high fibre and low energy contents) and lack of the constant supply of grasses and legumes. Increasing the efficiency with which forage is digested by the ruminal micro-organisms has been the subject of extensive investigations for over a century. Forage digestibility has been improved by several biotechnological products: ionophores, direct fed microbial products and enzymes. In the past two decades, the application of exogenous fibrolytic enzymes (EFE) has demonstrated to have the potential to increase forage utilization by rumen microbes, improve production efficiency and reduce nutrient excretion.

In vitro methods are both reliable and useful for comparative purposes identifying EFE that may have a positive effect with regard to production responses. After identification of their potential to increase the gas production (GP) at 24 hours, two EFE (Abo 374 and EFE 2) and one microbial yeast preparation were tested on four different substrates using organic matter digestibility (*in vitro* true digestibility) and fermentation characteristics (*in vitro* GP system). The different feed substrates were lucerne hay, wheat straw, wheat straw treated with urea and commercial concentrate diet. Results from the *in vitro* evaluations showed that EFE significantly enhanced *in vitro* DM degradability and GP profiles, with Abo 374 being the best treatment. The addition of the EFE was found to increase *in vitro* nutrient disappearances of different quality forages and the concentrate diet. However the cumulative GP at 48 hours was not correlated to the MPS of the GP residues. The MPS was significantly improved in the first half-period of incubation with EFE effects using the *in vitro* filter bag procedure. With the GP system, Abo 374 significantly increased MPS of the concentrate diet determined on residues of GP ($P < 0.0001$), but no EFE effects were detected amongst the forage substrates. The observed variations of MPS responses may be related to the microbial lysis with long periods of incubations and poor recovery of purine derivates with the Zinn & Owen (1986) analysis procedures. These findings suggested that the improvements in cumulative GP, synthesis of microbial protein and disappearance of DM and CP were likely obtained through a combined effect of direct enzyme hydrolysis and synergy between EFE and ruminal fibrolytic enzymes. On the basis of these results, Abo 374 was selected and consequently tested in another parallel *in vitro* and *in situ* investigation using a 1:1 mixed substrate of lucerne hay and wheat straw.

Abo 374 significantly improved the GP profiles and *in vitro* DM and NDF disappearance of the mixed substrate. However no correlation was found between the *in vitro* MPS and the cumulative GP at 48 hours. *In situ* disappearance of feed nutrients (DM, NDF and CP) with Abo 374 was similar to control. This may be due to the small number of sheep used in the study and the relatively high coefficient of variation associated with measuring ruminal digestion. In addition, Abo 374 significantly increased the *in situ* MPS, measured as purine derivates. The enhancement of GP profiles associated with the increase of *in situ* MPS and disappearance both *in vitro* and *in situ* of DM and NDF resulted from the addition of Abo 374 to the mixed substrate of

lucerne hay and wheat straw. This EFE appeared to have a stimulatory effect to initiate the primary microbial colonization and the release of digestion products that attract additional bacteria to the site of digestion. Findings of this investigation revealed that this EFE can efficiently affect the degradation of CP in addition to the enhancement of the disappearance of DM and NDF fractions to subsequently stimulate MPS. It could be speculated that positive effects of Abo 374 were due to the improvement of direct hydrolysis, microbial attachment and stimulation of the rumen microbial population and synergistic effects between exogenous and endogenous fibrolytic enzymes. It appears that the use of EFE in ruminant diets is limited by the variability in responses as also reported from literature. Sometimes, study results are reported with no information regarding enzyme type, concentration and activity, substrate specificity or with known enzyme activities measured at temperatures and pH levels different from the rumen. Rumen milieu can also influence EFE activity, making their responses on feed intake, digestibility and production traits somewhat inconsistent to predict in ruminant systems. Further research is therefore required regarding the digestibility and economical potential of Abo 374 when added to forage, concentrate, or total mixed diets with a large number of ruminants before this enzyme should be made available to commercial ruminant farmers.